STEROID SECRETORY PROFILE AND STEROIDOGENIC ACUTE REGULATORY (StAR) mRNA EXPRESSION IN CULTURED BOVINE ADRENOCORTICAL CELLS.

BY

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DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own. Work performed by others as part of collaborative studies are indicated in the acknowledgement section

Moira Ruth Nicol

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Abstract

ACTH is the principal factor stimulating the formation and release of cortisol from the adrenal cortex and is controlled, in part, by the negative feedback inhibition of cortisol. Investigation into the kinetics of cortisol production by cultured bovine adrenocortical cells in response to ACTH-treatment and the relationship between the cortisol secretion and the expression of the steroidogenic acute regulatory (StAR) mRNA was undertaken. ACTH (10nM) treatment of BAC cells on day 3 of culture displayed an increase in the rate of cortisol output over the initial 6 hours, $P<0.05$ at each time point compared with untreated cells, followed by a marked decline in the secretion rate thereafter. The decline in the cortisol secretion rate was not due to a decrease in the number of cells as the protein content of the culture wells did not vary by more than 10% over the 12 hour period. Nor was the decrease in cortisol secretion a result of serum-deprived culture as a comparable response was seen in serum-replete conditions. HPLC analysis of the culture medium revealed the secretion of four main steroids; cortisol, corticosterone, cortisone and 11β-hydroxyandrostenedione. Cortisol was found to account for ≥50% of the total steroid output by BAC cells at each of the time points studied. The total steroid output increased over the initial 6 hours (6.1nmol/10^6 cells at 6 hours) followed by a decline in levels to 4.4nmol/10^6 cells at 12 hours. Thus, the decline in cortisol secretion was not due to the production of another steroid in preference to cortisol. Various concentrations of ACTH also invoked a similar pattern of cortisol secretion as did treatment of BAC cells with angiotensin II. Interestingly, no decline in cortisol secretion was found when BAC cells were treated with forskolin or 8Br-cAMP. Receptor desensitisation was ruled out as the cells responded to a second ACTH-treatment. The response to the second ACTH-treatment produced a significant increase in cortisol output compared with the original response at the 8 and 12 hour time points, $P<0.05$. In addition the cells responded to a series of 12 hour ACTH treatments through 72 hours, with a constant level of cortisol being maintained. The protein levels over the 72 hour time course remained constant.

The immunoactivity of ACTH was found to be reduced after an overnight incubation at 37°C. ACTH, 0.01 and 1nM, showed a significant reduction ($P<0.05$) in activity when placed on BAC cells after an overnight incubation compared with ACTH prepared freshly on the day. ACTH at 10 and 100nM showed no significant reduction in activity. The concentration of 10nM ACTH in the culture medium was reduced by 77% after a 12 hour incubation at 37°C, $P<0.01$, in the absence of BAC cells. A 17% reduction in concentration was found in the presence of BAC cells, though this was not significant compared with the original concentration.

The expression of StAR mRNA was used as a marker for the acute regulation of steroidogenesis. Northern hybridisation revealed the presence of two StAR transcripts, 3.0 and 1.8kb. The expression of the StAR transcripts was found to be highest in freshly isolated cells, with both untreated and ACTH-treated cells producing similar amounts. By day 3 of culture the levels of StAR mRNA had decreased and day 3 was found to be the optimum time for the study of StAR mRNA expression. Upon ACTH-treatment of BAC cells the two transcripts show a biphasic response with a peak at 6 hours (10-fold increase over untreated) followed by a decline to almost untreated levels thereafter ($P>0.05$). A similar biphasic response was found with various ACTH concentrations and AngII-treatment. Forskolin-treatment showed a peak in StAR expression around 4 hour with a decline in levels, although these were still significantly raised over untreated cells at 8 hours. 8Br-cAMP showed no peak in expression with the levels of StAR mRNA remaining constant over the 12 hour period.

The use of conditioned medium on BAC cells suggested the presence of an inhibitory substance secreted into the medium. To ascertain if cortisol was exerting a local negative feedback effect, cortisol itself was added to the culture medium. A significant increase in the cortisol output over and above that present in the medium was found at 4 and 8 hours of ACTH-treatment. Cortisone was also found to have no inhibitory effect on the secretion of cortisol by BAC cells. Inhibition of the steroid pathway at various points prevented the production of any steroids; however an increase in the expression of the StAR transcripts was found. The reason for this increase in StAR expression is unknown at present.

In conclusion, ACTH-treatment of BAC cells produced a decrease in the secretion rate of cortisol, paralleled by a decline in StAR mRNA expression at around 6 hours. A series of experiments were conducted to investigate this observation. A decline in the immunoactivity of ACTH may contribute to this, but probably only at lower concentrations. More interestingly, evidence was found for the possible presence of a local negative feedback mechanism in cultured BAC cells. The identity of the factor(s) involved remains unresolved, though direct effects of cortisol and cortisone were ruled out.

Abbreviations

3β-HSD	3 beta-hydoxydehydrogenase
11β-HSD	11 beta hydroxysteroid dehydrogenase
17β-HSD	17 beta hydroxydehydrogenase
ACTH	adrenocorticotrophin
Adx-red	adrenodoxin reductase
AngII	angiotensin II
Ala	alanine
AmpB	amphotericin B
apo B	apolipoprotein B
apo E	apolipoprotein E
Arg	arginine
ARTISt	arachidonic acid-related thioesterase involved in steroidogenesis
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
AT_1	angiotensin type 1
BAC	bovine adrenocortical cells
bp	base pair
8Br-cAMP	8 bromo cyclic adenosine monophosphate
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
C/EBPs	CCAAT/enhancer binding proteins
CO_2	carbon dioxide
cpm	counts per minute
CPSR1	controlled processed serum replacement 1
CRE	cAMP response element
CREB	CRE binding
CRH	corticoprophin releasing hormone
CYP11A	cytochrome P450 side chain cleavage
CYP11B1	cytochrome 11β-hydroxylase
CYP17	17α-hydroxylase
CYP21	21-hydroxylase
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dbcAMP	dibutyrl cyclic adenosine monophosphate
DBD	DNA binding domain
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DHAS	dehydroepiandrosterone-sulphate
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid

dTTP	deoxytyrosine triphosphate
EBSS	Earle's balanced salts solution
EDTA	ethylenediaminetetraacetic acid
G-protein	guanyl nucleotide binding protein
GABA	gamma-amino-n-butyric acid
GDP	guanosine diphosphate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GRs	glucocorticoid receptors
GREs	glucocorticoid response elements
GTP	guanosine triphosphate
His	histidine
H_2O	water
HCl	hydrochloric acid
HDL	high density lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl CoA
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
hsp	heat shock protein
$InsP_2$	phosphotidylinostiol 4,5-bisphosphate
$InsP_3$	inositol 1,4,5 trisphosphate
kb	kilo bases
KCL	potassium chloride
kDa	kilodaltons
LCAH	lipoid congenital adrenal hyperplasia
LDL	low density lipoprotein
Leu	leucine
LH	luteinising hormone
Lys	lysine
MC2-R	melanocortin 2 receptor
Met	methionine
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MOPS	3-[N-morpholino]propanesulonic acid
MSH	melanocyte-stimulating hormone
Na_2HPO_4	sodium phosphate
NaCl	sodium chloride
NAD	α-nitotinamide adenine dinucleotide
NADH	α-nitotinamide adenine dinucleotide (reduced form)
NADP	α-nitotinamide adenine dinucleotide phosphate
NADPH	α-nitotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide

O_2	oxygen
OLC	ouabain-like compound
PBR	peripheral benzodiazepine receptor
PEG	polyethylene glycol
Phe	phenylalanine
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
POMC	preopiomelanocortin
Pro	proline
P/S	penicillin/streptomycin
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RNase	ribonuclease
RIA	radioimmunoassay
rpm	revolutions per minute
RT-PCR	reverse transcription polmerase chain reaction
SAP	steroidogenic activator protein
SCAP	SREBP cleavage activating protein
SCC	side-chain cleavage complex
SCP2	sterol carrier protein 2
SD	standard deviation
SDS	lauryl sulfate, sodium salt
Ser	serine
SERBP-1a	sterol regulatory element binding protein 1a
SF-1	steroidogenic factor-1
SR-B1	scavenger receptor, class B, type 1
SSC	3M sodium chloride 0.3M sodium citrate buffer
StAR	steroidogenic acute regulatory protein
START	StAR related transfer domain
TAE	tris acetate buffer
TE	tris/ ethylenediaminetetraacetic acid buffer
Tris	tris(hydroymethyl)aminomethane
Trp	tryptophan
TTP	thymidine triphosphate
Tyr	tyrosine
UV	ultraviolet
Val	valine
v/v	volume per volume
w/v	weight per volume

zf	zona fasciculata
zfr	zona fasciculata/reticularis
zg	zona glomerulosa
zr	zona reticularis

CHAPTER .1 INTRODUCTION

1.1 The Adrenal Gland

The anatomy of the adrenal gland was first described by Bartholomeo Eustachius in 1563 although little was known about its function (Thorn, 1968). The necessity of functional adrenal glands for survival was discovered in the 1850s based on observations by Addison of patients with destructive disease of the adrenal and Brown-Séquard provided experimental evidence using adrenalectomised animals (Orth, 1992). By the 1930s it was recognised that that the effects of adrenal insufficiency could be divided into two categories: those due to electrolyte imbalance and those arising from altered carbohydrate metabolism. In 1932 Cushing documented the presence of small pituitary adenomas in patients with adrenocortical hyperfunction (Orth 1992). We now know of its various functions affecting numerous body systems.

1.1.1 Gross anatomy of the adrenal gland

In mammals the adrenal glands are situated one on each side of the vertebral column, on the superior pole of each kidney. The shape and size of the adrenals varies between species from almost spherical in rats to more flattened and triangular in humans and cattle. In the human and bovine species, the left gland is crescent shaped and the right is more pyramidal. The size of the gland varies with the size of the animal, the human gland measures about 20-30mm in width, 40-60mm in length, 2-5mm in thickness and weighs about 4 grams. The gland is enclosed by several layers of connective cells surrounded by collagen fibers, which form the capsule. Relative to its size it has a larger vascular and autonomic nerve supply compared to most other organs. The adrenal has a unique structure comprising of two functionally and embryologically distinct tissues, the inner medulla composed of neurectoderm-derived chromaffin cells surrounded by an outer cortex of mesodermic origin (figure 1.1). The utility, if any, of having them together in one discrete organ is not

known. In some species, e.g. amphibians and certain fish, two separate organs are found (Orth, 1992).

1.1.1.1 The adrenal medulla

The adrenal medulla consists almost entirely of chromaffin cells. These are irregular shaped polyhedral cells organised in cords or small clumps and surrounded by nerves, connective tissue and blood vessels. Within the chromaffin cells are numerous chromaffin granules containing the catecholamines, noradrenaline and adrenaline. In addition, chromaffin vesicles contain numerous transmitters, neuropeptides and proteins which may be released with the catecholamines (Ehrhart-Bornstein, 1998).

1.1.1.2 The adrenal cortex

The adrenal cortex is divided into three concentric zones (figure 1.2). The outermost region is the zona glomerulosa (zg) which lies directly underneath the capsule and secretes predominantly the mineralocorticoids. The secretory cells of the zg are arranged in irregular ovoid clumps. The cytoplasm of the secretory cells contains much smooth endoplasmic reticulum, numerous mitochondria and triglyceride droplets, all essential for steroid production.

The zona fasciculata (zf) is the intermediate and broadest of the three zones, comprising approximately 75% of the cortex, and secretes predominantly the glucocorticoids. It consists of parallel cords of secretory cells disposed at right angles to the capsule and separated by fine strands of supporting tissue containing capillaries. The cytoplasm is even more rich in smooth endoplasmic reticulum and lipid droplets than the zg.

The zona reticularis (zr)is the innermost zone responsible for secretion of small quantities adrenal androgens and glucocorticoids. The zr consists of an irregular network of branching cords and clumps of granular cells separated by numerous wide

diameter capillaries. The cells are much smaller than those found in the zf and contain fewer lipid droplets (Burkitt, 1993).

The degree of demarcation of the three zones varies between species, the zf and zr of the bovine adrenal cortex merge to form a single layer, whilst the bovine zg is clearly defined from the inner zones. In contrast, the human zg is poorly demarcated and cells from the zf can be observed directly under the capsule.

Several hypotheses exist to explain the formation and maintenance of the adrenocortical zones. The zonal hypothesis proposes that each zone contains differentiated cells with its fixed complement of steroidogenic enzymes and functions as a self-contained unit. Each zone is responsible for replenishment of cells following local cell death (Chester-Jones, 1957). Other theories are less rigid and suggest migration of the cells through the cortex during their life span. The cell migration theory proposes that a stem cell layer exists beneath the capsule which contributes cells to the zg. These cells migrate through each of the zones, changing steroidogenic function as the zone requires, and then dying within the zr (Zajicek, 1986). Hornsby proposed the steroid gradient hypothesis which argues that the centripetal vascular system created a gradient of a particular substance across the cortex (possibly corticosterone) which regulates the expression of the steroidogenic enzymes. This theory is dependent on the concept that the cells of each zone arise from the same basic cell type, having temporary and morphological differences. This concept is supported by a study showing that cultured adrenocortical cells from each zone quickly reverted to the same steroidogenic phenotype (Hornsby, 1983).

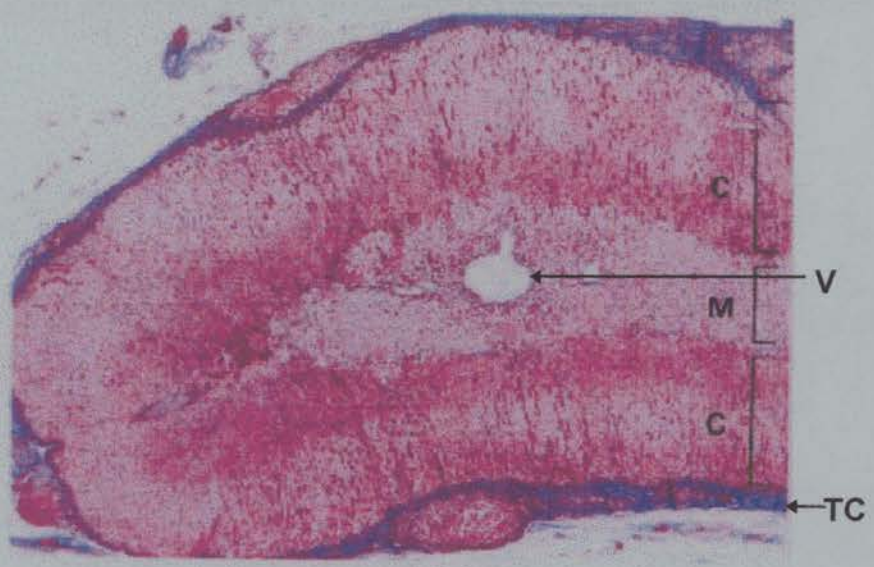

Figure 1 1: Human Adrenal gland. (x12). The outer cortex (C) and a pale stained inner medulla (M) can be seen. A dense fibrous tissue capsule (TC), stained blue, surrounds the gland and provides external support. A prominent vein (V) is characteristically located in the center of the medulla (Burkitt, 1993).

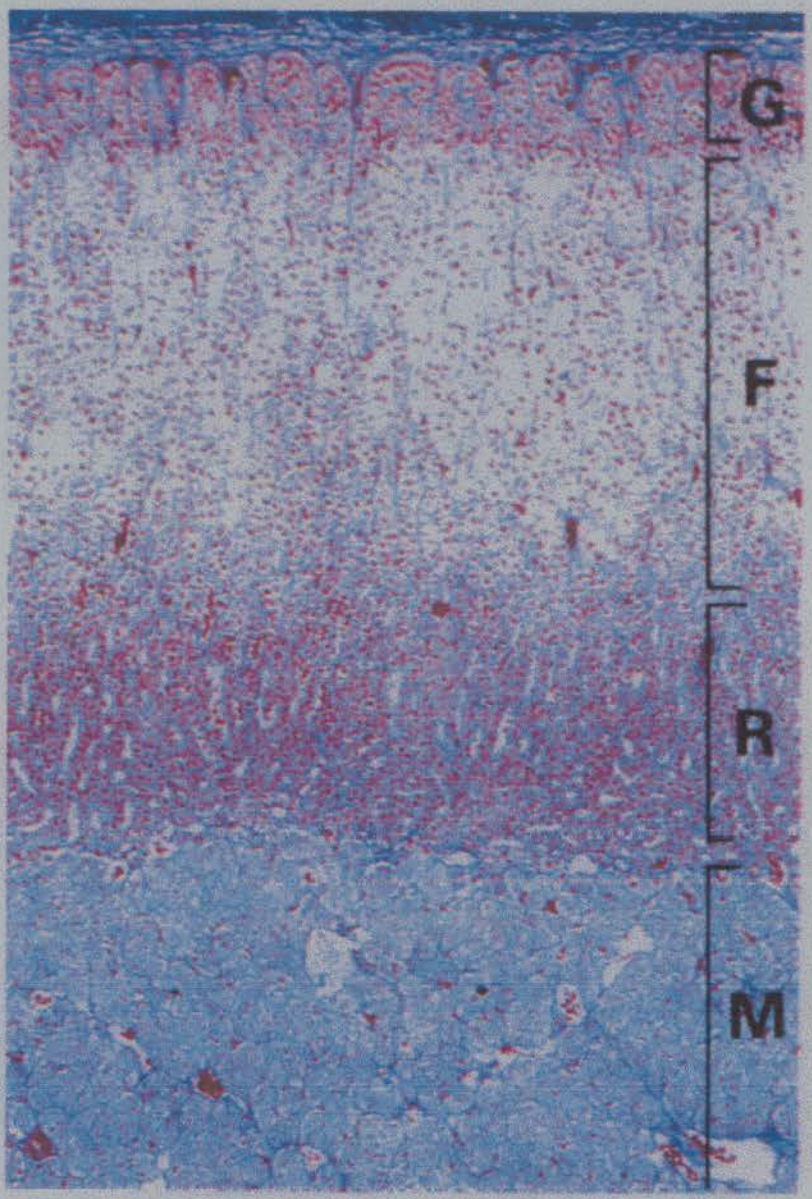

Figure 1.2 : The morphological zonation of the human adrenal cortex. (x20)The zona glomerulosa (G) lies beneath the capsule and consists of secretary cells arranged in rounded clumps. The intermediate zona fasciculata (F) consists of parallel cords of secretary cells disposed at right angles to the capsule. The zona reticularis (R) lying adjacent to the medulla (M) consists of small closely-packed cells arranged in irregular cords (Burkitt, 1993).

1.1.2 Blood supply and innervation

Blood is supplied to the mammalian adrenal by numerous small arteries branching from the aorta, inferior phrenic and renal arteries. Cortical arteries and arterioles branch into capillary beds within the cortex. These vessels arise directly from the capsule, initially forming an anastomotic network around the cells of the zona glomerulosa, then continuing as longitudinal capillary sinusoids between the cells of the zona fasciculata. Then vessels finally open into a plexus of large bore sinusoids that surround the cells of the zona reticularis and, hence coalesce into veins at the corticomedullary junction. The organisational pattern of the cortical vasculature ensures that every steroidogenic cell is closely associated with at least one cortical sinusoid. This is believed to be necessitated by the need to support the oxygen demand made by the cytochrome P450 mediated hydroxylations of the stimulated adrenal cortex (Bassett, 1997). Medullary arteries and arterioles penetrate the cortex without branching then form capillary beds in the medulla. Blood from both the cortical and medullary veins empties through a large central vein, leaving the adrenal via the renal vein (left gland) or the vena cava (right gland) (Orth, 1992).

Early descriptions of the microanatomy of the adrenal cortex concluded that the cortex was not directly innervated, with the nerve supply to the medulla passing directly through the cortex without branching or synapsing with any adrenocortical cells. However it is now generally accepted that the mammalian adrenal cortex receives a rich nerve supply, with nerve terminals in direct contact with cortical cells (Ehrhart-Bornstein, 1998).

1.2 Hypothalamic-pituitary-adrenal (HPA) axis

Adrenal glucocorticoid release is regulated by hormonal interactions between the hypothalamus, pituitary and the adrenal. The hypothalamus produces corticotrophin-releasing hormone (CRH) a 41 amino acid peptide synthesised in the parvocellular

portion of the paraventricular nucleus. CRH enters the hypophysial portal blood system and is transported to the anterior pituitary where it stimulates receptors on pituitary corticotrophs to synthesis and secrete adrenocorticotrophin (ACTH). ACTH in turn stimulates the adrenal cortex to produce glucocorticoids (figure 1.3) (Orth, 1992). Cortisol secretion is closely regulated by ACTH and there are three mechanisms of neuroendocrine control: 1) episodic secretion and the circadian rhythm of ACTH, 2) stress responsiveness of the HPA-axis and 3) feedback inhibition by cortisol of ACTH secretion (Aron, 2000a). The circadian rhythm is superimposed on episodic secretion; it is the result of central nervous system events that regulate both the number and magnitude of CRH and ACTH secretory episodes. In humans the release of ACTH and glucocorticoids throughout the day follows a circadian rhythm. The highest levels are found in the morning and the lowest levels in the late evening. Circadian rhythms in plasma glucocorticoids has also been shown in monkey (Holaday, 1977), sheep (Fulkerson, 1979) and cow (Thun, 1981). The circadian rhythm can be altered by changes in sleep pattern, light-dark exposure and physical or physiological stress such as surgery or depression. ACTH and cortisol secretion are also responsive to physical stresses. Stress responses originate in the central nervous system and increase CRH. CRH release occurs in response to physical stress (pain, hypoxia, surgery, exercise) or psychological stress (fear, anticipation). Other factors such as arginine vasopressin and catecholamines can indirectly regulate steroidogenesis through the HPA in a pulsatile, diurnal rhythmic manner. The third major regulator of ACTH and cortisol secretion is that of feedback inhibition. Glucocorticoid feedback inhibition occurs at both the pituitary and hypothalamus and involves two distinct mechanisms: fast and delayed feedback inhibition (Aron, 2000b). Fast feedback inhibition of ACTH secretion is rate dependent, i.e. it depends on the rate of increase of the glucocorticoids. This phase is rapid, and ACTH secretion diminishes within minutes following an increase in glucocorticoid levels. This fast feedback phase is transient and is though to occur via actions on the cell membrane rather than via glucocorticoid receptors (Aron, 2000b). Delayed feedback inhibition further suppresses CRH and ACTH secretion, thus with

continued glucocorticoid administration ACTH levels continue to decrease and become unresponsive to stimulation. The ultimate effect of continued glucocorticoid administration is suppression of CRH and ACTH release and atrophy of the zonae fasciculata and reticularis as a result of ACTH deficiency (Aron, 2000b). Delayed feedback appears to act via the glucocorticoid receptor (see below), reducing the synthesis of the messenger RNA for pro-opiomelanocortin, the precursor for ACTH (Aron, 2000a). (Figure 1.3).

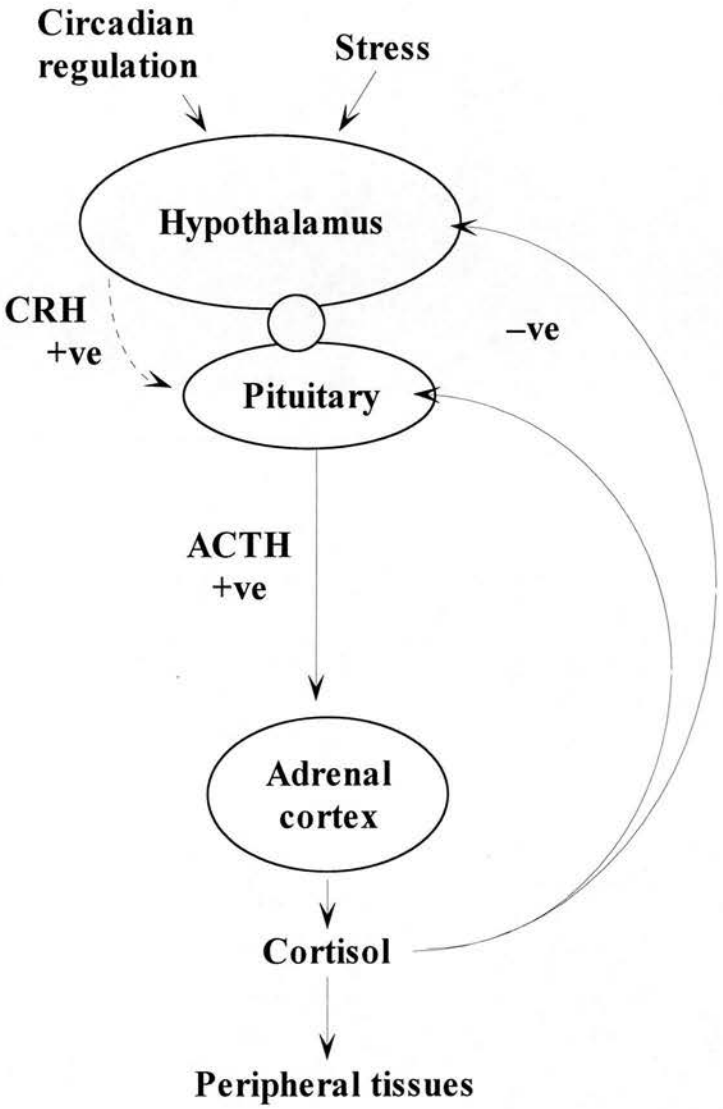

Figure 1.3 Hypothalamic-pituitary-adrenal axis Diagrammatic representation of the HPA axis. Corticotrophin releasing hormone (CRH) and ACTH stimulate (+ve) and cortisol inhibits (-ve) the appropriate gland.

1.2.1 The intra-adrenal CRH-ACTH axis

A complete CRH-ACTH system exists within the adrenal, with the adrenal medulla and/or intraadrenal nerves as the source of CRH and adrenal medullary chromaffin cells as target for this local releasing hormone and the source of ACTH (Ehrhart-Bornstein, 1998). Data from different studies have demonstrated that the CRH influences adrenocortical steroidogenesis independently from the HPA-axis, CRH exerts an effect on hypophysectomised rats (Bornstein, 1990) and calves (Jones, 1992). CRH immunoreactivety has been observed in human and bovine adrenal medulla (Suda, 1986).

A direct effect of CRH on adrenocortical function seems unlikely, as CRH was shown to have no effect on isolated adrenocortical cells (van Oers, 1992). ACTH immunoreactivity was found in extracts of rat (Mazzocchi, 1993) and human (Suda, 1986) adrenal medulla and in the adrenal venous effluent of hypophysectomised calves in response to splanchnic stimulation (Jones, 1992). In addition, adrenal fragments composed mainly of chromaffin cells released ACTH in response to high concentrations of CRH (Andreis, 1992). In patients receiving ACTH replacement therapy for proven pituitary ACTH deficiency, subsequent CRH administration induced a significant increase in cortisol levels that was preceded by a rise in ACTH levels (Fehm, 1988). Thus suggesting that the adrenal medulla is a source of extrapituitary ACTH that maybe stimulated by CRH.

1.2.2 Mechanism of steroidogenic action of ACTH in the adrenal

The actions of ACTH can conveniently be divided on temporal grounds; into acute actions which take place over a few minutes/hours and the long-term effects which require a period of hours or even days to become manifest. Both the acute and the chronic responses are hormonally controlled via activation of adenylate cyclase resulting in an increase in intracellular levels of cAMP and activation of the protein kinase A pathway (Simpson, 1988), (Sala, 1979).

1.2.2.1 Acute action of ACTH

This effect generally involves an increase in cholesterol supply to the inner mitochondrial enzyme CYP11A, which catalyses the first step in adrenal steroid biosynthesis. In addition, ACTH increases the rate of transport of cholesterol across the inner mitochondrial membrane, this process is discussed in detail later (section 1.6)

1.2.2.2 Chronic action of ACTH

The longer term requirement for ACTH in maintaining steroid levels in adrenocortical cells was first established from studies in hypophysectomised rats. Purvis et al showed that the levels of both mitochondrial and microsomal P450 enzymes fall dramatically after hypophysectomy, but were restored following ACTH administration. (Purvis, 1973). It therefore appears that ACTH acts chronically on the adrenal cortex to regulate the levels of several if not all of the components of the steroidogenic pathway (Simpson, 1983). It was subsequently shown that ACTH acts to maintain the level of steroid output by inducing steroidogenic enzyme mRNA synthesis and translation via a cAMP-dependent mechanism (Simpson, 1988). ACTH is known to increase the expression of CYP11A (DuBois, 1981), CYP17 (Zuber, 1985), (Zuber, 1986), (McCarthy, 1983), 3β-HSD (Le Roy, 2000), CYP21 (Funkenstein, 1983) and CPY11B1 (Kramer, 1983). Each steroid hydroxylase gene requires its own unique biochemical system for cAMP-dependent transcription (Waterman, 1997). Discrete regulatory DNA elements and specific DNA-binding proteins that participate in this highly regulated, cAMP-dependent gene expression have been identified (Waterman, 1997). Investigation of the biochemical basis of the ACTH-dependent transcription regulation of the steroid hydroxylase genes in the adrenal has uncovered both traditional (CREB) and nontraditional (SF-1, NGFI-B, Sp1, ASP, PBX) cAMP-dependent transcription factors. For the nontraditional factors it remains necessary to elucidate the coupling of PKA activity to the functions of these proteins (Waterman, 1997).

1.3 Second messenger pathways

Steroidogenic tissue can respond almost immediately to a stimulatory hormone. It is well established that the interaction of a steroidogenic hormone, such as ACTH, with its specific receptor at the cell surface can trigger the cAMP messenger system. The activation of other second messenger systems, such as the phosphoinositol or arachidonic acid systems, may occur depending on the stimulus and intensity of stimulation.

1.3.1 Adenylate cyclase

The earliest signalling mechanism identified in adrenocortical cells was the adenylate cyclase system (Sutherland, 1958). In mammals, adenylate cyclase is a single polypeptide that resides in the plasma membrane of cells. Its role is to catalyse the production of the intracellular second messenger molecule, 3',5'-cyclic monophosphate (cAMP) from adenosine triphosphate (ATP). When a hormone e.g. ACTH, binds to the relevant cell surface receptor, activation of adenylate cyclase is not a direct process (figure 1.4). Activation of the receptor in turns causes the activation of a guanyl nucleotide-binding proteins (G-protein), which regulates the activity of adenylate cyclase (Gilman, 1995). The action of G-protein on adenylate cyclase activity is either stimulatory or inhibitory depending on the G-protein involved (G_s or G_i respectively). In the inactive state G-protein exists as a complex of three polypeptides, α, β and γ, and has guanosine diphosphate (GDP) bound to a single GDP-binding site. On activation of the receptor, the GDP is released in exchange for guanosine triphosphate (GTP), causing the breakdown of the G-protein into a free α subunit bound to GTP and a β/γ complex that does not dissociate further. The free α subunit-GTP diffuses to adenylate cyclase, where it binds and causes activation, resulting in the subsequent release of cAMP. The process ends when intrinsic GTPase of the α subunit hydrolyses the bound GTP to GDP and the α subunit reforms with the β/γ complex. (Gilman, 1995).

cAMP further transmits the intracellular signal by activating a cAMP-dependent protein kinase, protein kinase A (PKA), leading to subsequent phosphorylation events. The inactive form of PKA is a tetramer consisting of two catalytic and two regulatory subunits. cAMP binds to the regulatory subunits, leading to their dissociation from the catalytic subunits. The free catalytic subunits are then enzymatically active and able to phosphorylate their target proteins (Doskeland, 1993). Intracellular cAMP is inactivated by conversion to 5'-AMP, a reaction catalysed by the enzyme phosphodiesterase (Hancock, 1997).

The effects of increased intracellular cAMP levels in response to ACTH include phosphorylation of cholesterol esterase and cholesterol ester synthetase, leading to the respective activation and inactivation of these enzymes and a consequent increase in the availability of free cholesterol from cholesterol esters, enhancement of cholesterol transport into the mitochondria and facilitation of cholesterol binding to CYP11A (Orth, 1992).

Increases in cAMP activates the transcription of specific target genes. The regulatory region of the gene activated by cAMP contains a short DNA sequence called the cAMP response element (CRE) (Hancock, 1997). CREs were originally identified as cis-acting elements that confer transcriptional activation in response to elevated cAMP levels. The CRE sequence is recognised by a specific gene regulatory protein called CRE-binding (CREB) protein (Dooley, 1999). CREB is phosphorylated, by PKA on a single serine residue, in response to elevated cAMP. Phosphorylation allows the CREB to interact efficiently with the transcriptional co-activator called CREB-binding protein to stimulate transcription of cAMP target genes (Dooley, 1999).

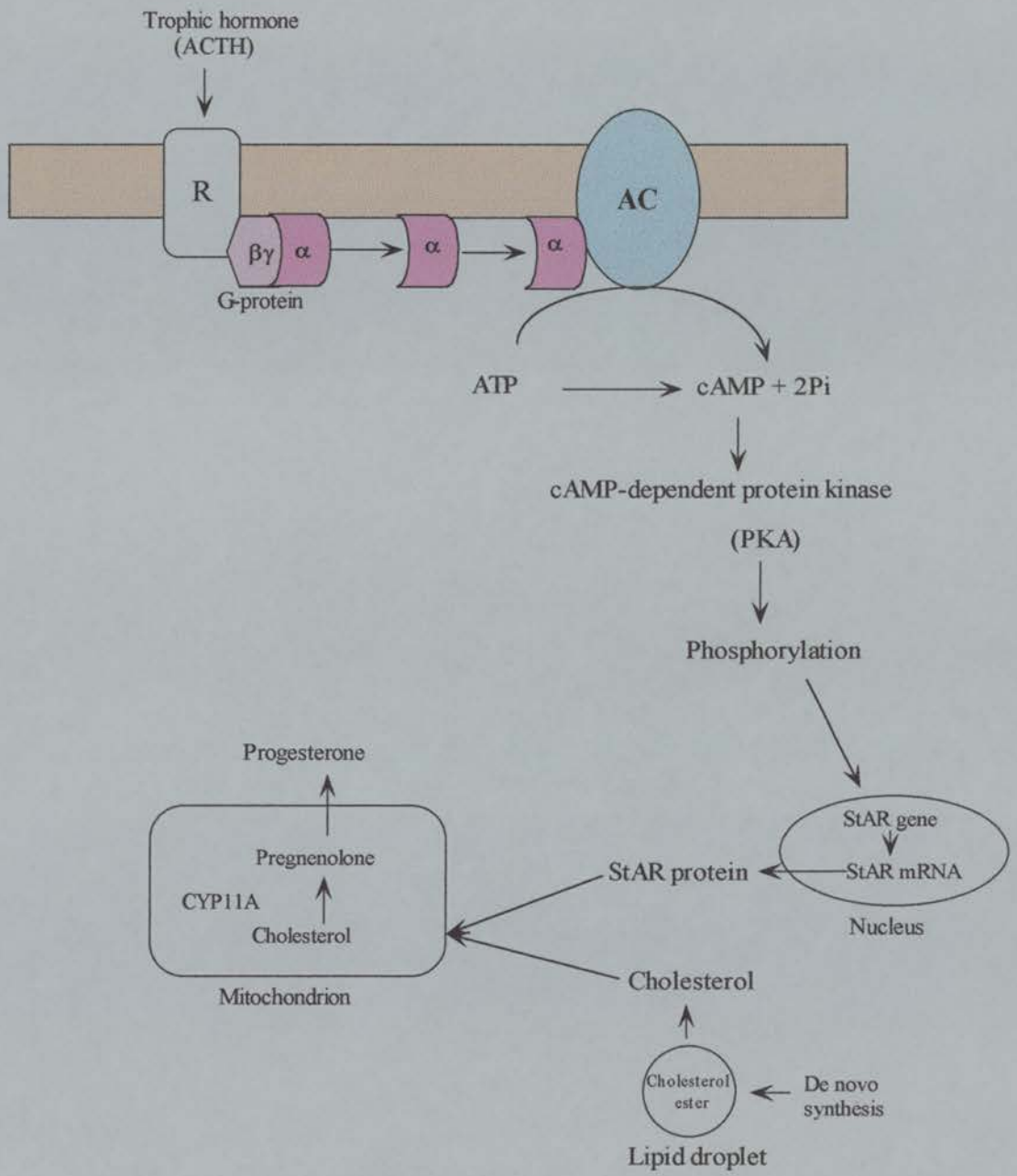

Figure 1.4 Hormonal activation of adenylate cyclase. Binding of ACTH promotes the interaction of the receptor (R) with a G-protein. The activated G-protein α subunit then dissociates from the receptor and stimulates adenylate cyclase (AC), which catalyses the conversion of ATP to cAMP. The rise in cAMP activates PKA leading to phosphorylation, resulting in the synthesis of StAR protein and initiation of steroidogenesis.

1.3.2 Signalling through membrane phosphoinositides

Between 2 and 8 % of the lipids in eukaryotic membranes are inositol containing lipids. One of the most widespread pathways of intracellular signaling is based on the use of second messengers derived from the hydrolysis of the membrane bound phospholipid, phosphatidylinositol 4,5-bisphosphate ($InsP_2$). These messengers are generated by a membrane transduction process comprising three components: a receptor, a coupling G protein and phospholipase C (PLC). Stimulation of cell surface receptors initiates the hydrolysis of $InsP_2$ producing two important metabolites; inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) which serve as second messenger signals (figure 1.5). DAG acts by stimulating protein kinase C (PKC) and $InsP_3$ releases calcium from internal stores (Berridge, 1989). $InsP_3$ is recycled by sequential phosphorylation/dephosphorylation back to inositol and DAG is recycled through conversion to phosphatidic acid and cytidine diphospodiacylglycerol, which combines with free inositol to reform phosphatidylinositol. Thus, although the stimulation of PLC results in the increased breakdown of $InsP_2$ the net effect is to increase phosphoinositide turnover, rather than to decrease phosphoinositide mass (Berridge, 1989).

The activation of the phosphoinositol/PLC system in bovine and rat zona glomerulosa (Kojima, 1985a), (Farese, 1984) as well as bovine zona fasciculata/reticularis cells (Clyne, 1992), (Bird, 1989), (Walker, 1991b) in response to angiotensin II (AngII) has been demonstrated. Using BAC cells as a model it has been demonstrated that AngII works through an inhibitory guanine nucleotide-binding regulator of adenylate cyclase (G_i)-and a stimulatory G protein regulator of phospholipase C (G_q)-coupled AngII-type 1 receptor. This leads to inhibition of adenylate cyclase and stimulation of PLC and protein kinase C (Sasaki, 1991), (Ouali, 1992), (Clyne, 1993a).

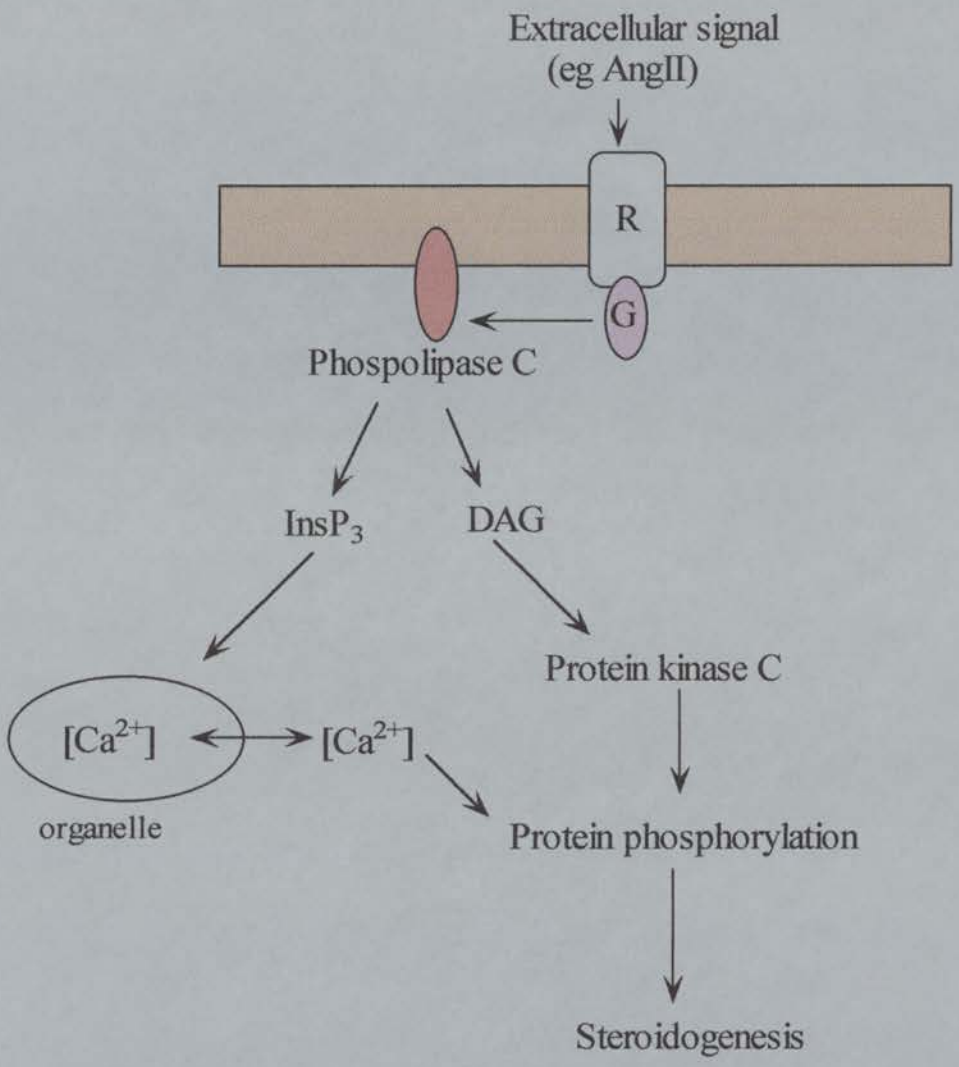

Figure 1.5 Activation of phospholipase C. Binding of an extracellular signal, such as AngII, promotes the interaction of the receptor (R) with a G-protein (G). The activated G-protein stimulates phospholipase C which hydrolyses $InsP_2$ to produce the second messengers $InsP_3$ and diacylglycerol (DAG).

1.3.3 Arachidonic acid

Growing evidence has suggested a role for arachidonic acid in steroid production in the adrenal gland. Arachidonic acid can be released from the 2-position on the glycerol backbone of phospholipids by calcium mediated activation of phospholipase A_2 (PLA_2) or alternatively by activation of phospholipase C. It can function itself as an intracellular mediator or be further metabolised through one of three pathways, lipoxygenase, cyclooxygenase or cytochrome P450-dependent epoxygenase, to produce various metabolites (Cooke, 1999).

An adrenocorticotrophic hormone-induced 43-kDa protein has been purified and cloned in rat adrenal cells, named arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) (Finkielstein, 1998). ARTISt has been shown to act as an intermediary in the regulation of PLA_2 activity by cAMP and therefore plays a role in the activation of steroidogenesis by helping to mediate the release of arachidonic acid (Maloberti, 2000).

Lipoxygenase metabolites of arachidonic acid have been shown to stimulate steroidogenesis in bovine adrenal cells. The 15-lipoxygenase pathway was found to be activated by low concentrations of ACTH, at which no increase in cellular cAMP was observed (Yamazaki, 1996), (Wang, 2000). It has been postulated, therefore, that at low concentrations of ACTH-stimulation of adrenal cells, arachidonic acid metabolites are the principal second messengers, whereas at high concentrations of ACTH stimulation cAMP is the principal messenger (Mele, 1996).

In rat and bovine glomerulosa cells it was observed that AngII stimulated the release of arachidonic acid and that inhibition of this release prevented aldosterone production (Natarajan, 1990), (Kojima, 1985b). It has therefore been suggested that the mechanism for ACTH and AngII stimulated steroidogenesis may involve an increase of intracellular arachidonic acid release.

The mechanism by which arachidonic acid and its metabolites regulate hormone-induced steroidogenesis is unclear (figure 1.6). It has been postulated that arachidonic acid and its metabolites regulate the transfer of cholesterol from the outer to the inner mitochondrial membranes. Therefore several studies have investigated the role of arachidonic acid in StAR protein regulation. Using inhibitors of arachidonic acid an essential role for arachidonic acid in steroid biosynthesis and StAR protein and gene expression was demonstrated in LH-stimulated MA-10 cells (Wang, 1999a).

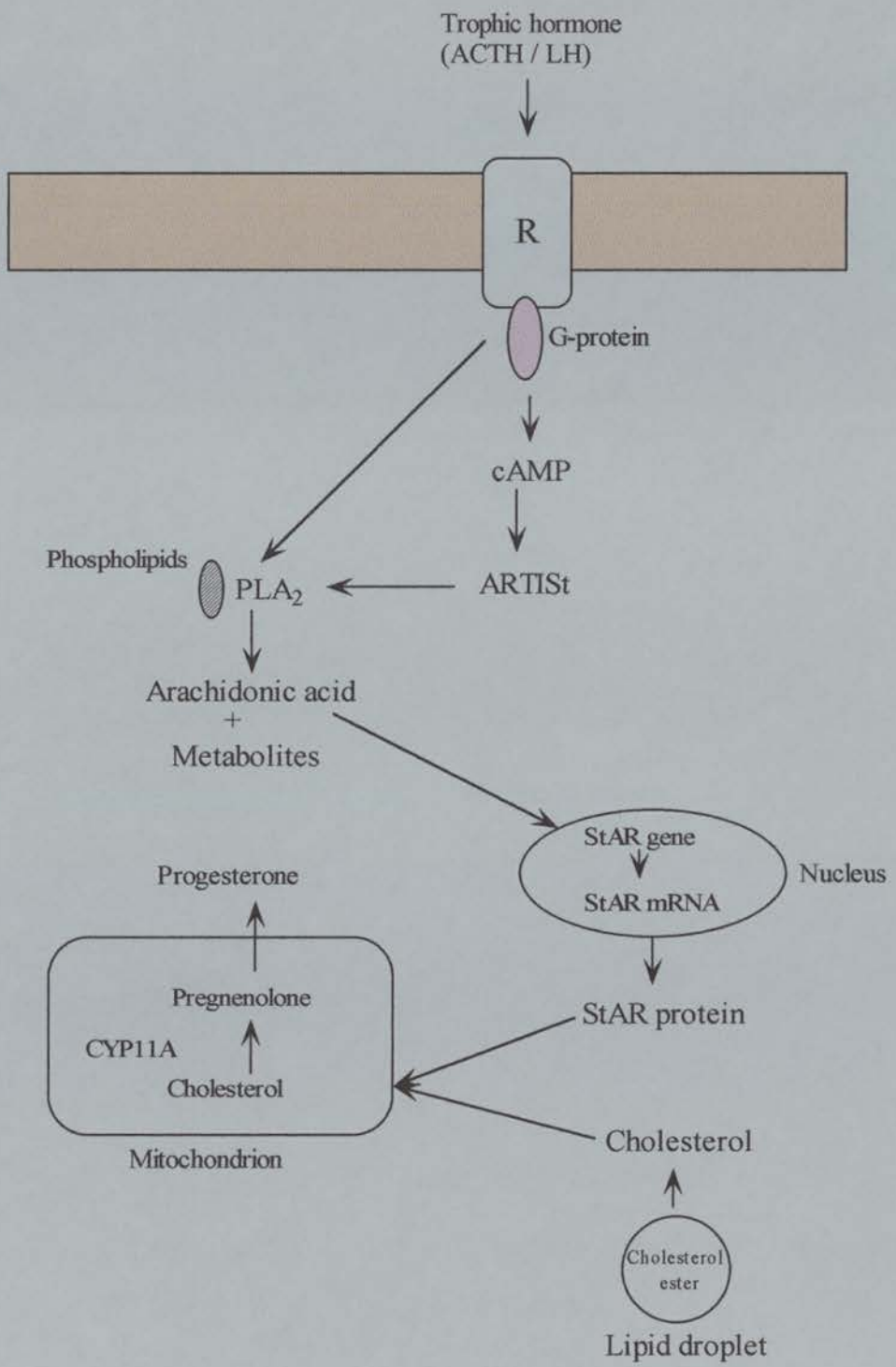

Figure 1.6 Proposed hormonal activation of arachidonic acid. Trophic hormone stimulation induces arachidonic acid release from phospholipids, either dependently or independently of cAMP. The nature of this pathway remains to be elucidated. Adapted from (Wang, 1999b).

1.4 The Adrenal Steroids

1.4.1 Structure of steroids

The adrenal cortex secretes the steroid hormones, essential regulators of many physiological processes within the body. Endogenous steroids are produced from cholesterol, which is a complex molecule containing 27 carbon atoms and possesses a ring structure common to the steroid hormones, the cyclopentanophenanthrene nucleus. This nucleus consists of three cyclohexane rings and one cyclopentane ring joined to each other by common sides (Figure 1.7) (Orth, 1992).

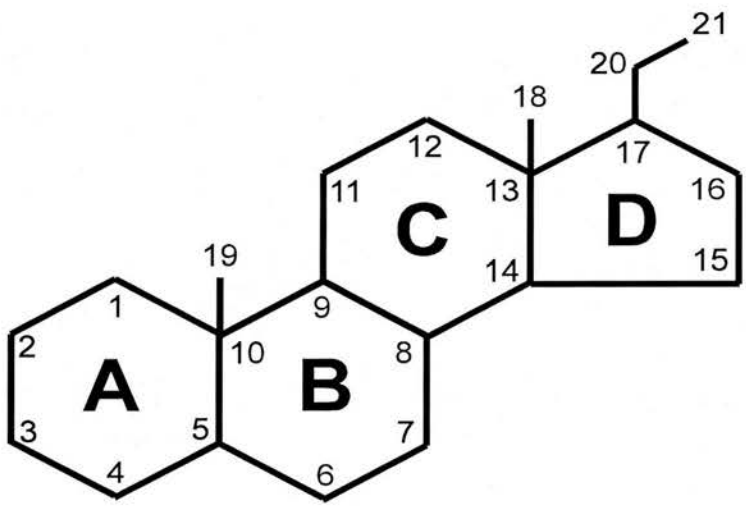

Figure 1.7 : Basic steroid ring structure. The four rings are identified by letters. Each carbon atom is numbered as shown. Adapted from (Brooks, 1979)

The adrenocortical steroids are of two structural types: those that have a 2-carbon side chain attached at position 17 of the D ring and contain 21 carbon atoms (C-21 steroids), and those that have a keto or hydroxyl group at position 17 and contain 19 carbon atoms (C-19 steroids) (Schulster, 1976). The C-19 steroids have androgenic activity and the C-21 steroids have glucocorticoid and mineralocorticoid activity.

The main adrenal steroids are those with mineralocorticoid or glucocorticoid activity, although some sex steroids, (androgens and estrogens) are also secreted. The secretion rates and biological activity of the major steroid secreted by the human adrenal cortex are shown below (table 1.1)

		relative biological potency		
steroid	**secretion rate**	**glucocorticoid***	**mineralocorticoid***	**androgen****
cortisol	100	100	100	-
corticosterone	5	30	160	-
aldosterone	0.75	-	30000-50000	-
DOC	1.2	-	1000	-
androstenedione	3	-	-	20
DHEA	125	-	-	10
DHEA.S	8	-	-	20
testosterone	0.06	-	-	100

Table 1.1 Secretion rates and biological activities of the major steroids secreted by the human adrenal cortex. Secretion rates are given relative to cortisol (around 15-30mg/day in humans). The biological activities expressed relative to cortisol* or testosterone**. (Brooks 1979)

1.4.2 The mineralocorticoid

Mineralocorticoids play a critical role in regulating the concentrations of minerals, particularly sodium and potassium, in extracellular fluids. The principal steroid with mineralocorticoid activity is aldosterone. The major target of aldosterone is the distal tubule of the kidney where it's principal function is to regulate water and electrolyte balance. Aldosterone acts by facilitating the reabsorption of sodium and the excretion of potassium and hydrogen ions in the distal renal tubule. Aldosterone also has similar effects on sweat glands, salivary glands and the colon. The major net effect is

to conserve body sodium by stimulating its resorption (or absorption in the case of the colon), conservation of water following conservation of sodium. Aldosterone output is controlled by the renin-angiotensin system. Renin, a protein made in the kidney, converts angiotensinogen to angiotensin I, which is subsequently converted to an active peptide, angiotensin II (AngII). AngII interacts with cell surface receptors in the zona glomerulosa stimulating aldosterone synthesis (Brooks, 1979).

1.4.3 Adrenal androgens

Androstenedione, dehydroepiandrosterone (DHEA) and its sulfate (DHAS) analog are the main C19 steroids secreted by the adrenal cortex. They have minimal intrinsic adrenrogenic activity, and they contribute to androgenicity by their peripheral conversion to the more potent androgens, testosterone and dihyrotestestosterone. Although DHEA and DHAS are secreted in greater quantities, androstenedione is qualitatively more important as it is more readily converted peripherally to testosterone (Aron, 2000b).

1.4.4 The Glucocorticoids

In most mammals the principal glucocorticoid secreted is cortisol, whereas, due to a lack of the CYP17 enzyme, rodent adrenals secrete corticosterone as the major glucocorticoid. Glucocorticoids have a diverse array of actions with effects on intermediary metabolism, the nervous system and some process related to reproduction (Orth, 1992).

1.4.4.1 Effects on intermediary metabolism

As the name suggests glucocorticoids regulate glucose metabolism. In the fed state the effects of glucocorticoids are minimal. However, during fasting, glucocorticoids stimulates several processes that collectively serve to increase and maintain blood glucose concentrations. In the liver glucocorticoids increase hepatic glucose

production by increasing substrate availability and stimulating release of glucogenic amino acids from peripheral tissue, such as skeletal muscle. Glucocorticoids also directly activate key hepatic gluconeogenic enzymes, such as glucose-6-phosphate and phosphoenolpyruvate carboxylinase (Orth, 1992). Glucocorticoids also alter carbohydrate metabolism by inhibiting peripheral glucose uptake in muscle and adipose tissue. Glucocorticoids acutely activate lipolysis in adipose tissue with the release of glycerol and free fatty acids. This is partially due to direct stimulation of lipolysis by glucocorticoids, but is also contributed to by decreased glucose uptake and enhancement of the lipolytic hormones by glucocorticoids (Aron, 2000b).

1.4.4.2 Effects on other tissues and functions

Glucocorticoids have potent anti-inflammatory and immunosuppressive properties (Orth, 1992). One of the principal glucocorticoid effects is on immune cells trafficking to and from the peripheral circulation (Fauci, 1974), causing a redistribution of lymphocytes from the intravascular compartment to the spleen, lymph nodes, thoracic duct and bone marrow. Glucocorticoids also prevent local inflammatory responses by inhibiting the movement of cells and fluid from the intravascular space (Aron, 2000b). As a consequence glucocorticoids have been used to treat inflammatory conditions such as rheumatoid arthritis, and as adjunction therapy for conditions such as autoimmune diseases (Orth, 1992).

Glucocorticoids accelerate the development of a number of systems and organs in fetal and differentiating tissues (Aron, 2000b). Examples of these development-promoting effects include increased surfactant production in the fetal lungs (Odum, 1988) and accelerated development of hepatic and gastrointestinal enzyme systems (Aron, 2000b).

Glucocorticoids also have an effect on numerous other tissues. For example, in connective tissue, excess glucocorticoids inhibit fibroblasts, leading to loss of collagen and connective tissue, thus resulting in thinning of the skin. Glucocorticoids

readily enter the brain, and although their function in the central nervous system is unknown, their excess or deficiency may have profound effects on behavior and cognitive function (Orth, 1992).

1.4.5 The glucocorticoid receptor

Glucocorticoids travel via the blood stream to their target cells, enter the cells by simple or facilitated diffusion, and then bind to specific glucocorticoid receptor. Most tissues in the body appear to possess glucocorticoid receptors, including the adrenal cortex (Loose, 1980). The glucocorticoids receptor is a ligand-induced transcription factor that belongs to the steroid/thyroid receptor superfamily (Beato, 1995). The steroid hormone receptor superfamily represents the largest known family of transcription factors described for eukaryotes (Tsai, 1994). Amino acid sequences show that the receptors can be divided into several domains (figure 1.8). At the N-terminal end is a variable region known as the A/B domain, which is involved in gene activation. The C domain contains two zinc fingers and is responsible for DNA recognition and for dimerisation. Downstream of the C region, a variable hinge region, the D domain. This domain may contain sequences responsible for localisation of the receptor to the nucleus. Ligand binding is to a large E domain. This region is approximately 250 amino acids in length and is also responsible for association with heat shock proteins, hsp90, hsp70 and hsp56 (Pratt, 1993) and dimerisation. At the C-terminal end is the last region called the F domain, but no function has been assigned to this area of the protein (Tsai, 1994).

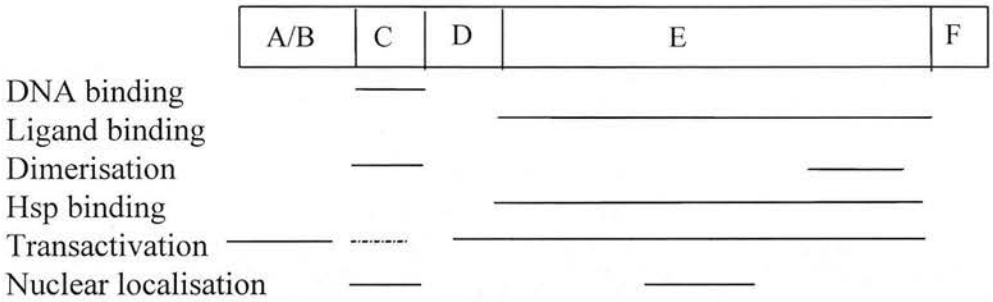

Figure 1.8 Functional domains of steroid receptors. The structure of steroid receptors can be divided into six domains, A, B, C, D, E and F. the function of each domain is indicated by the solid lines.

Glucocorticoid receptors (GRs) are mainly localised within the cytoplasm (Wikstrom, 1987), (Htun, 1996). The cytoplasmic receptor, when bound by glucocorticoid, undergoes an activation or 'transformational' change, dissociates from the hsp complex and exposes the DNA binding domain (DBD) region which allows translocation from the cytoplasm to the nucleus (reviewed in (Reichardt, 1998). Once GRs arrive within the nucleus, the activated receptor influences gene transcription either by binding to glucocorticoid response elements (GREs) or by interacting with other transcription factors (Reichardt, 1998). The resulting proteins elicit the glucocorticoids response, which may be inhibitory or stimulatory depending on the specific gene and tissue affected. Intermediary steroids such as progesterone, 11-deoxycortisol and deoxycorticosterone may function as partial antagonists, in the presence of more active glucocorticoids. However the physiologic role of these hormones in glucocorticoid action is probably negligible as they circulate in low concentrations.

The syndrome of familiar glucocorticoid resistance, first described in 1976, is a disorder characterised by hypercortisolism without Cushingoid features (Vingerhoeds, 1976). Abnormalities of the GR number, affinity for glucocorticoids, stability and translocation into the nucleus have been described (Hurley, 1991), (Karl, 1996), (Karl, 1993), (Malchoff, 1993).

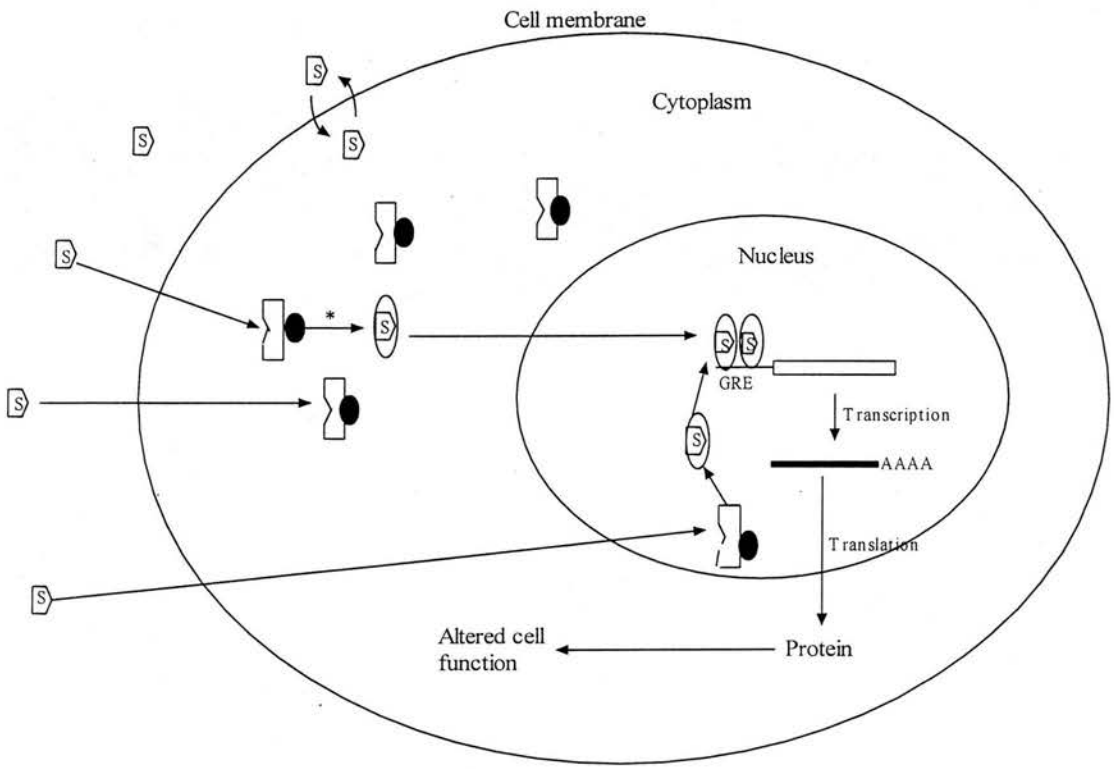

Figure 1.9 A simplified model of glucocorticoid action. Glucocorticoids (S) enter the cell by diffusion and bind to specific receptors, with associated hsp (●). The binding of the glucocorticoid causes the receptor to undergo a transformation step (*). The activated receptor is directed into the nucleus, where it binds to a specific glucocorticoid-response element (GRE). This initiates transcription of the corresponding mRNAs and the translation of these mRNAs are proteins that alter cell function. See text for more detail.

1.4.5 Disorders of adrenal steroids

The main disorders of adrenocortical function are hyperfunction or hypofunction. Hyperfunction occurs when there is excessive secretion of cortisol (Cushing's syndrome), or aldosterone (primary aldosteronism). Androgens may also be secreted in excess (Brooks, 1979).

Deficient adrenal production of glucocorticoids or mineralocorticoids results in adrenocortical insufficiency, which is either the consequence of destruction or dysfunction of the cortex (primary adrenocortical insufficiency or Addison's disease) or secondary to deficient pituitary ACTH secretion (secondary adrenocortical insufficiency) (Orth, 1992). The etiology of primary adrenocortical insufficiency has changed over time. Prior to 1920, tuberculosis was the major cause of adrenocortical insufficiency. Since 1950, autoimmune adrenalitis with adrenal atrophy has accounted for about 80% of cases. It is associated with a high incidence of other immunologic and autoimmune endocrine disorders, such as HIV (Aron, 2000b). Primary adrenocortical insufficiency, or Addison's disease, is rare, with a reported prevalence of 110 per million population in the United Kingdom, and is more common in females (Aron, 2000b). Clinical signs and symptoms do not become manifest until at least 90% of the adrenal tissue is destroyed. Hyperpigmentation is the classic physical finding and is due to the high circulating levels of ACTH resulting from the lack of cortisol suppression of the feedback inhibition. Cortisol deficiency causes weakness, fatigue, anorexia, nausea and vomiting, hypotension, hyponatremia and hypoglycemia. Mineralocorticoid deficiency produces renal sodium wasting and potassium retention and can lead to severe dehydration, hyperkalameia, hyponatremia and acidosis (Aron, 2000b).

Secondary adrenocortical insufficiency due to ACTH deficiency is most commonly a result of exogenous glucocorticoid therapy. Pituitary or hypothalamic tumors are the most common causes of naturally occurring pituitary ACTH hyopsecretion. ACTH

deficiency is the primary event and leads to decreased cortisol and adrenal androgen secretion. Aldosterone secretion remains normal in the majority of cases (Aron, 2000b). The manifestations of glucocorticoid deficiency are similar to those for primary adrenocortical insufficiency; however, since aldosterone secretion is usually normal the manifestations of mineralocorticoid deficiency are absent (Orth, 1992).

Glucocorticoid excess or Cushing's syndrome is caused by an excess of circulating cortisol, which is high at all times i.e. no diurnal rhythm. It is most commonly iatrogenic, resulting from chronic glucocorticoid therapy. Spontaneous Cushing's syndrome is caused by abnormalities of the pituitary or adrenal or may occur as a consequence of ACTH secretion by nonpituitary tumors (ectopic ACTH syndrome) (Tsigos, 1995). Cushing's syndrome is classified as either ACTH-dependent or ACTH-independent. The ACTH-dependent types, Cushing's disease or ectopic ACTH syndrome, are characterised by chronic ACTH hypersecretion, which results in hyperplasia of the adrenal zonae fasciculata and reticularis. Resulting in increased secretion of cortisol, androgens and deoxycorticosterone. Cushing's disease is the most frequent type of Cushing's syndrome accounting for 70% of reported cases (Orth, 1995). ACTH-independent Cushing's syndrome may be caused by a primary adrenal neoplasm (adenoma or carcinoma) or nodular adrenal hyperplasia. In these cases the excess cortisol suppresses pituitary ACTH secretion (Orth, 1995). Mild to moderate obesity, typically involving the face and trunk, is the most common presenting feature of Cushing's syndrome.

The most prominent metabolic abnormalities reflect the glucocorticoid action. About 75% of patients have glucose intolerance and many of these have hyperglycemia (Tsigos, 1995). Protein breakdown is accelerated and the carbon chains of the liberated amino acids may be converted to glucose, the nitrogen is also lost in the urine leading to a negative nitrogen balance. This catabolic effect not only causes osteoporosis and muscle wasting but also thinning of the skin. The tendency to bruising and purple striae, most obvious on the abdominal wall, are probably due to

this thinning (Orth, 1995). The mineralocorticoid effects of cortisol leads to urinary retention of sodium, with hypertension occurring in about 75% of cases. High circulating levels of androgens may account for the common findings of greasy skin with acne vulgaris, and for the hirsutism and menstrual problems in women (Tsigos, 1995).

1.5 Biosynthesis of steroid hormones

Two major types of enzymes are involved in steroid biosynthesis: 1) the cytochrome P450s and 2) steroid dehydrogenases. The main enzymes involved in human and bovine adrenal steroid biosynthesis are listed below (table 1.2).

Enzyme	Gene symbol	Location	Protein length (aa)
P450ssc	CYP11A	mitochondrial	482 (481)
3β-hydroxysteroid dehydrogenase	HSD3B1 HSD3B2	microsomal	371 (371)
P450c17	CYP17	microsomal	508 (509)
P450c21	CYP21	microsomal	494-495 (496)
P450c11β	CYP11B1	mitochondrial	479 (479)

Table 1.2 Genes encoding the major steroidogenic enzymes of the adrenal cortex. Numbers in brackets represent the protein length in the bovine species.

Steroidogenic P450s catalyse either a single hydroxylation at a specific position on the steroid or a series of consecutive hydroxylations which result in C-C bond cleavage (Hanukoglu, 1992). Each P450-catalysed hydroxylation has the following stoichiometry:

$$\text{Steroid-H} + \text{NADPH} + \text{H}^+ + \text{O}_2 \longrightarrow \text{Steroid-OH} + \text{NADP}^+ + \text{H}_2\text{O}$$

The first enzymatic step in the production of all steroids is the side chain cleavage of cholesterol to pregnenolone by CYP11A (figure 1.10). The reaction consists of three successive monooxygenations, hydroxylations at C-22, followed by C-20, and finally cleavage of the C-20,22 bond. This reaction takes place in the mitochondria. Reducing equivalents from mitochondrial NADPH are transferred by a flavoprotein (adrenodoxin reductase) to a ferredoxin protein containing an iron sulphur cluster (adrenodoxin), which then transfers the electrons to CYP11A. (Stocco, 1996). CYP11A is expressed in all three zones of bovine and human adrenal cortex. (Ishimura, 1997). The other components involved in the side chain cleavage of cholesterol, adrenodoxin and adrenodoxin reductase have also been localised in the matrix and inner mitochondrial membrane of bovine adrenocortical cells (Hatano, 1989), (Mitani, 1979).

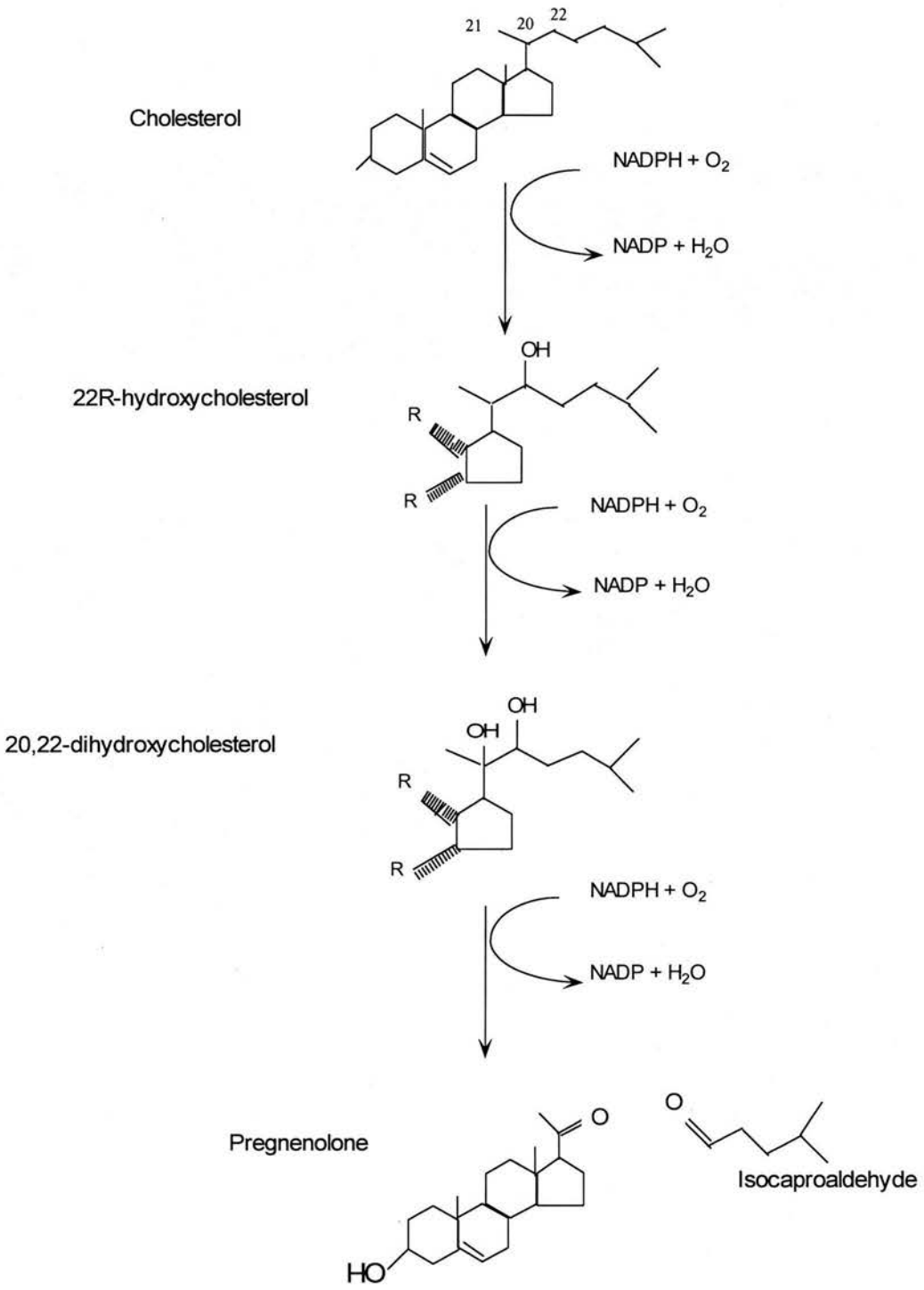

Figure 1.10 Reaction sequence for the conversion of cholesterol to pregnenolone by CYP11A.

Pregnenolone produced by this reaction leaves the mitochondria and is transported to the endoplasmic reticulum, where it undergoes further transformation. In the adrenal cortex of human, bovine, ovine and pigs pregnenolone is converted by CYP17 to 17α-hydroxypregnenelone (figure 1.11). This enzyme catalyses two distinct reactions: the 17α-hydroxylation of C21 steroids, a step required for cortisol production, and the cleavage of the C17-C20 bond of C21 steroids essential for androgen biosynthesis. Rodents such as rat, mouse hamsters and guinea pig synthesise corticosterone as the principal glucocorticoid due to the lack of CYP17 expression in the adrenal (Le Goascogne, 1991). CYP17 is present in the zona fasciculata and reticularis but not in the zona glomerulosa of bovine, and human adrenals (Ishimura, 1997).

Pregnenolone and 17-hydroxypregnenolone are converted to progesterone and 17-hydroxyprogesterone respectively by a two step reaction: dehydrogenation of 3β-hydroxy-5-ene-steroid and isomerization of 3-oxo-5-ene-steroid. This two step process is catalysed by 3 β-hydroxysteroid dehydrogenase (3β-HSD). 3β-HSD appears to be situated in the membrane of the smooth endoplasmic reticulum and is present in all three zones of the cortex (Ishimura, 1997). At least two types of 3β-HSD (types I and II) have been cloned in the human, although only the type II enzyme has been detected in the adrenal cortex (Labrie, 1994), whereas in the bovine types I, II, III and IV have been found (Morel, 1997).

CYP21, a microsomal enzyme, catalyses the conversion of progesterone and 17-hydroxyprogesterone to deoxycorticosterone and deoxycortisol respectively (figure 1.11). Immunohistochemical staining has found CYP21 expression in all three zones of the adrenal cortex (Ishimura, 1997). Congenital adrenal hyperplasia is the result of an inborn deficiency in CYP21 and results in steroid biosynthesis being channeled in the direction of androgen synthesis, resulting in the excessive production of the C19 steroids, DHEA and androstenedione. Androstenedione may be converted peripherally to testosterone causing virilisation (Aron, 2000b). In this condition the cortex is broadened and consists mainly of zr-type cells (Hanukoglu, 1992).

Deoxycortisol and deoxycorticosterone then leave the endoplasmic reticulum and enter the mitochondria for the final step in glucocorticoid production (figure 1.11). Here they are converted to cortisol and corticosterone respectively by CYP11B1. CYP11B1 is also located on the matrix side of the inner mitochondrial membrane and utilises reducing equivalents provided by adrenodoxin and adrenodoxin reductase, as described for CYP11A. (Ishimura, 1997).

In the cells of the zona glomerulosa a parallel series of events takes place. Aldosterone is synthesised from deoxycorticosterone by a two-step reaction : 11β-hydroxylation and aldehyde formation at the C18 position catalysed by CYP11B2 (Ishimura, 1997). CYP11B2 has many properties similar to CYP11B1 but the two enzymes are distinct molecular species encoded by different genes. The gene for CYP11B2 has been isolated from the adrenal cortex human (Kawamoto, 1992) and cow (Hashimoto, 1989). In the case of the bovine adrenal cortex, both aldosterone and cortisol are synthesised by CYP11B1, although the precise regulatory mechanism of this enzyme is not known (Ishimura, 1997).

Cortisol and corticosterone are converted to their corresponding inactive forms, cortisone and 11-dehydrocorticosterone respectively, by 11β-hydroxysteroid dehydrogenase (11β-HSD) (Mazzocchi, 1998).There are two isoenzymes of 11β-HSD, type 1 and type 2 (11β-HSD1 and 11β-HSD2) (Mazzocchi, 1998). 11β-HSD1 is a bi-directional enzyme with low affinity for glucocorticoids and is found in the liver, testis, lung and kidney (Funder, 1996). 11β-HSD2 is a unidirectional enzyme with high affinity for glucocorticoids and is expressed in high concentrations in the kidney, adrenal and colon (Romero, 2000). 11β-HSD plays a key role in controlling the access of glucocorticoids to their receptors and protecting the nonselective mineralocorticoid receptor from glucocorticoid excess (Mazzocchi, 1998).

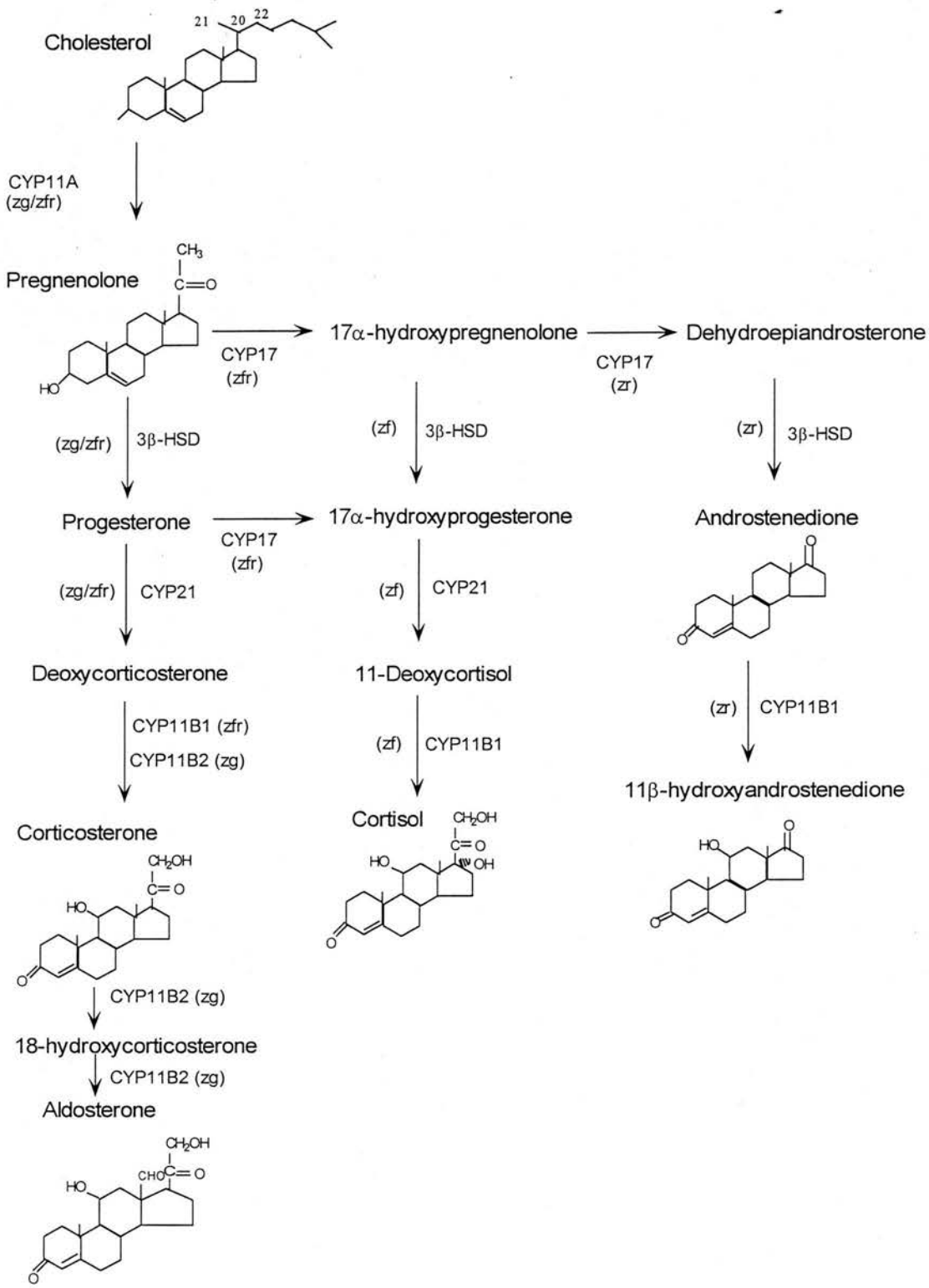

Figure 1.11 Diagrammatic representation of the steroid pathway in the human adrenal. Showing the steroids produced by the zona glomerulosa (zg); fasciculata (zf) and reticularis (zr).

1.6 Acute regulation of steroidogenesis

Trophic hormones activate a chain of reactions that lead to free cholesterol being transported to the mitochondria where it is converted to pregnenolone and then by further enzymatic steps to glucocorticoids or mineralocorticoids. This process is dependent on the supply and intracellular transfer of cholesterol.

1.6.1 Sources of cholesterol

Cholesterol is an essential component of cell membranes, the cholesterol within the membranes is not static and a constant cycling of cholesterol between the plasma membrane and other intracellular compartments such as the Golgi, lysosomes and endoplasmic reticulum exists. Steroidogenic cells convert cholesterol into steroid hormones in response to hormonal stimulation. This process is unrelated to cholesterol homeostasis within the cells. Therefore the steroidogenic cells require a supply of cholesterol that exceeds the constitutive cellular requirements. There are two principal sources of cell cholesterol.

1.6.1.1 Endogenous cholesterol biosynthesis.

Cholesterol biosynthesis from acetate or other cholesterol precursors produces free cholesterol or cholesterol esters if esterified by acetyl:cholesterol acyltransferase. Cholesterol synthesis begins with the synthesis of acetoacetyl CoA from two acetyl CoA molecules. A third acetyl CoA molecule is added by the enzyme hydroxy-3-methylglutaryl-CoA synthase to form hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then converted to cholesterol through various intermediaries including mevalonate, squalene and lanosterol. Cholesterol acts as a feedback inhibitor of HMG-CoA reductase to regulate the intra- and intercellular levels of cholesterol (figure 1.12) (Schulster, 1976). The genes encoding HMG-CoA reductase were found to contain a sterol-regulatory element in the proximal promoter region (Brown, 1997). A family of transcription factors called sterol regulatory element binding

protein (SREPB) was found to bind to this element and promote the expression of the HMG-CoA and other genes, such as the LDL-receptor, involved in cholesterol biosynthesis (Brown, 1997). Cholesterol interacts with a protein on the endoplasmic reticulum and Golgi named SREBP activating protein (SCAP) that in turn controls the activity of a protease that cleaves the precursor of SREBP (Brown, 1997), (Brown, 2000). When cholesterol levels are adequate, the protease is not activated and therefore SREBP is not released and does not migrate to the nucleus. As a result, expression of the genes for HMG-CoA reductase is not induced. When the cholesterol levels are low, the SREBP precursor is cleaved, SREBP migrates to the nucleus and the expression of the HMG-CoA reductase gene is promoted (Brown, 2000). Thus, cholesterol levels in a cell are sensed by the SCAP-SREBP pathway to feedback regulate the biosynthesis of cholesterol.

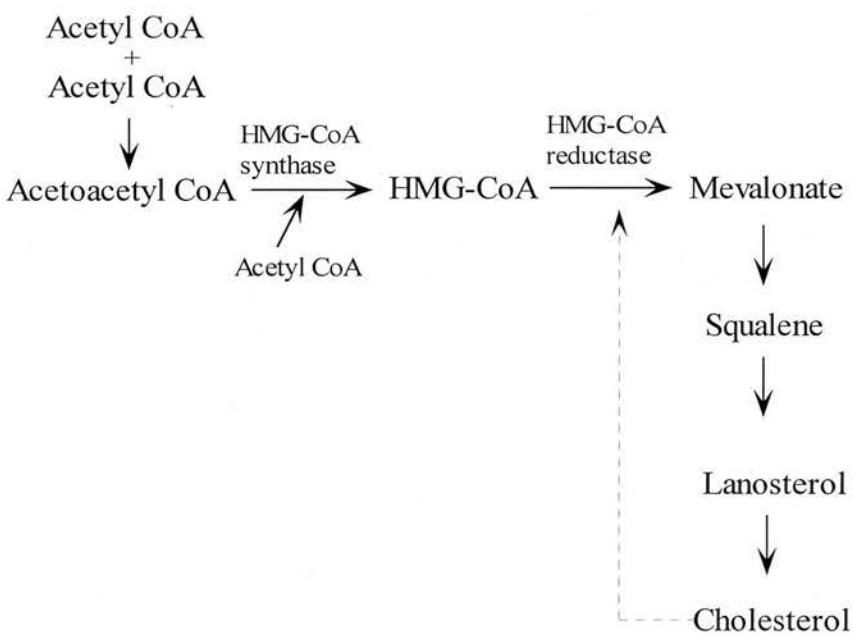

Figure 1.12 Biosynthesis of cholesterol. The adrenal cortex can synthesize cholesterol from acetate. In this synthesis the key enzyme is HMG-CoA reductase, which converts hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate. The dashed arrow indicates feedback inhibition of mevalonate formation by cholesterol

1.6.1.2. Uptake of cholesterol from plasma lipoproteins

Exogenous lipoproteins such as high density lipoproteins (HDL) or low density lipoproteins (LDL), carry and deliver both free and cholesterol esters to the cells (Sviridov, 1999). Cholesterol is taken up from plasma lipoproteins and subsequently stored in intracellular lipid droplets. Lipid droplets appear to be a morphological characteristic of steroidogenic cells, since they have been observed in all species studied to date (Nussdorfer, 1986).

In humans, the primary pathway for cellular cholesterol uptake involves the low density lipoprotein (LDL) receptor. These receptors function via endocytic uptake and lysosomal degradation of lipoprotein particles to release cholesterol and other lipids into the cell (Krieger, 1999). An alternative process, occurring with HDL, is the selective uptake pathway in which HDL cholesteryl ester is taken into the cell without the uptake and degradation of the HDL particle. The selective uptake pathway is active in a variety of mammalian cell types including human and bovine adrenal cells (Yaguchi, 1998), (Cherradi, 2001), but is particularity active in steroidogenic tissue of rats and mice (Pittman, 1987), (Acton, 1996). The biochemical mechanism of selective uptake is unclear.

1.6.2 Cholesterol transport

Once within the steroidogenic cell, cholesterol transport in response to hormone stimulation can be thought of as occurring in two stages. The first part of the process is the mobilization of cholesterol from cellular stores to the outer mitochondrial membrane, while the second part consists of the transfer of cholesterol from the outer to the inner mitochondrial membrane.

1.6.2.1 Cholesterol transport to the mitochondria

The factors and processes involved in the mobilisation of cholesterol to the outer mitochondrial membrane are thought to involve changes in cellular architecture and transport proteins. Studies have demonstrated that cholesterol transport to the mitochondria is dependent on an intact cytoskeleton (Feuilloley, 1996). The cytoskeleton is formed by three types of polymers, microtubules, microfilaments and intermediate filaments. Cytochalasin B and other inhibitors of microfilament and microtubule function have been found to block cholesterol transfer to the mitochondria in adrenal cells (reviewed in (Hall, 1997). Microtubules are required by adrenocortical cells for the transduction of the corticotrophic message of ACTH and cholesterol transport, whereas microfilaments control the metabolism of polyphosphoinositides as well as the transport of cholesterol and intermediate steroids from cytoplasm to the mitochondria. The intermediate filaments are involved in the stimulus-response coupling of certain corticotrophic factors such as AngII (Feuilloley, 1996).

A role for cellular cholesterol carrier or transport proteins was postulated in order to facilitate the movement of the hydrophobic cholesterol through the aqueous spaces and deliver them to specific organelles, such as the mitochondria. Several proteins, protein complexes and subcellular structures have been suggested to play a role in cholesterol trafficking (reviewed (Sviridov, 1999). These include clathrin-coated pits which are involved in trafficking cholesterol from the plasma membrane to the lysosomes and trans-Golgi network. Clathrin-coated pits are involved in LDL-receptor mediated endocytosis. After LDL binds to the LDL receptor, the ligand-receptor complex is sequestered into clathrin-coated pits (Krieger, 1999). The scavenger receptor class B, type 1 (SR-B1) mediates the selective uptake of HDL cholesteryl ester in transfected cells, thus suggesting that SR-B1 may be responsible for this activity in steroidogenic cells (Acton, 1996), (Temel, 1997). SR-B1 is most abundant in steroidogenic tissues utilising HDL-derived cholesterol for the synthesis of steroid hormones (Acton, 1996).

1.6.2.2 Cholesterol transport within the mitochondria

As mentioned above the first step in steroidogenesis is the conversion of cholesterol to pregnenolone, a reaction catalysed by CYP11A which resides on the matrix side of the inner mitochondrial membrane. Studies have shown that the true rate-limiting step effected by hormone treatment was the delivery of cholesterol from the outer to the inner mitochondrial membrane (Jefcoate, 1987), (Privalle, 1983), (Karaboyas, 1965). In accordance with this proposal was the finding that the hydroxylated analogs of cholesterol, which can readily diffuse across the mitochondrial membranes, were able to reach the inner mitochondrial membrane thus promoting steroidogenesis in the absence of trophic hormone stimulation (Lambeth, 1982), (Tuckey, 1989). These observations indicate that CYP11A was fully active and that the lack of cholesterol for steroid biosynthesis was the limiting factor. The two mitochondrial membranes are separated by an aqueous space through which the hydrophobic cholesterol must pass (figure 1.13). The aqueous diffusion of cholesterol is very slow and cannot account for the rapid increase in hormone-stimulated steroid production, necessitating a mechanism is required for the transport of cholesterol across this barrier (Phillips, 1987).

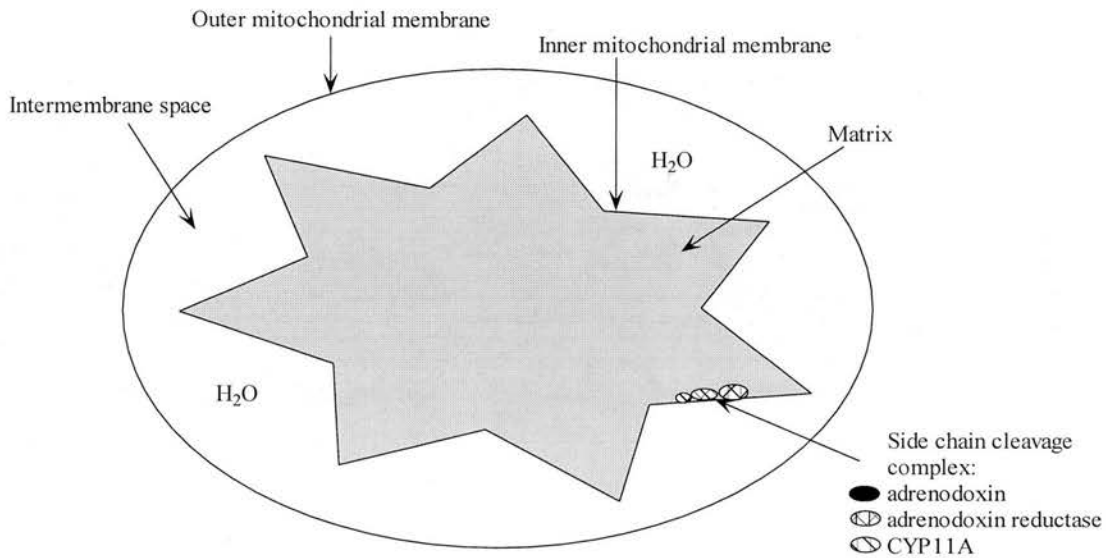

Figure 1.13 Graphic illustration of the mitochondrial compartments and components of the SCC complex. This diagram shows the aqueous space through which the relatively hydrophobic cholesterol has to pass to reach the side chain cleavage (SCC) complex.

Early studies demonstrated that ACTH-stimulated steroidogenesis was blocked by protein synthesis inhibitors such as puromycin and cycloheximide (Ferguson, 1962), (Garren, 1965). These studies also demonstrated that the step blocked by the inhibitors was distal to cholesterol ester hydolysis but proximal to side chain cleavage of cholesterol i.e. at the delivery of cholesterol to CYP11A (Davis, 1968) and had a rapid turnover time (Garren, 1965). It has been shown that the cycloheximide-sensitive step is located in the mitochondria (Simpson, 1966) and that the protein synthesis inhibitors had no effect on CYP11A (Arthur, 1976). It was later demonstrated that the delivery of cholesterol from the outer to the inner mitochondrial membrane was the step inhibited by cycloheximide (Privalle, 1983). Cholesterol may be delivered to a pre-steroidogenic pool in the presence of cycloheximide; however, pregnenolone production does not occur until the inhibitor is removed and the cells stimulated with trophic hormone (Stevens, 1993). As a

result of these observations, a search began for a hormone-sensitive, rapidly synthesised, highly-labile, cycloheximide-sensitive protein whose function was the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane.

1.7 Candidates for the transfer protein

A variety of candidates have been put forward as the protein involved in the acute regulation of steroidogenesis. These have included sterol carrier protein 2 (SCP2), steroidogenesis activator peptide (SAP), the peripheral benzodiazepine receptors (PBR) and the steroidogenic acute regulatory (StAR) protein.

1.7.1 Sterol carrier protein 2

SCP2 is a small, 13kDa protein, postulated to play a role in intracellular transport and metabolism of lipids such as cholesterol. SCP2, also known as non-specific lipid transfer protein, is found to be abundant in the liver and various steroidogenic tissues. SCP2 has been demonstrated to transfer cholesterol from lipid- containing vesicles to mitochondria and can increase pregnenolone production in isolated adrenal mitochondria (Chanderbhan, 1986). Chanderbhan also demonstrated that treatment of adrenal cells with anti-SCP2 antibody resulted in an inhibition of steroid production (Chanderbhan, 1986). The synthesis of SCP2 has been shown to be under the regulation of ACTH in the adrenal; however, this regulation only occurred after many hours of treatment and was not cycloheximide sensitive (Trzeciak, 1987). The exact mechanism of SCP2 action is not fully understood. One hypothesis is that SCP2 acts to transport cholesterol through the cytoplasm (reviewed in (Seedorf, 2000).

1.7.2 Steroidogenesis activator peptide.

SAP is a 3 kDa protein that was first purified from rat adrenals (Pedersen, 1983). SAP is found only in steroidogenic tissue. Its levels can be acutely increased by trophic hormone stimulation and these changes are blocked by cycloheximide. A role for SAP in cholesterol transfer within the mitochondria was demonstrated by addition of SAP to isolated mitochondria resulting in a 4- to 5-fold increase in steroid production (Pedersen, 1983). SAP shows a striking homology to a heat-shock protein called glucose-related protein (GRP 78). Heat shock proteins can act as molecular chaperones and allow proteins to unfold so they can be imported through contact points in mitochondria (Lill, 1996). However, a mechanism whereby SAP can transport cholesterol to the inner mitochondrial membrane has not yet been elucidated.

1.7.3 Peripheral benzodiazepine receptor

Benzodiazepines are widely used for their anxiolytic, anticonvulsant and hypnotic actions. These effects are mediated by the γ-aminobutyric acid (GABA) receptor located in the central nervous system (Pritchett, 1989). However, a second class of receptor has been identified in peripheral tissues, the peripheral benzodiazepine receptor (PBR) (Verma, 1989). PBR is a 18kDa membrane-spanning receptor found primarily on the outer mitochondrial membrane in peripheral tissue such as lung, liver and kidney. The greatest expression of PBR was shown to be in steroid-producing cells such as the adrenal, ovary and testis (Papadopoulos, 1993). Radioligand binding studies indicated that PBR was primarily associated with mitochondria (Basile, 1986), (Hirsch, 1989). PBR expression was found to be induced by ACTH in BAC cells (Besman, 1989). Disruption of the PBR gene in R2C rat Leydig tumor cells resulted in the reduction of steroid secretion, whereas introduction of the receptor back into cells resulted in a return to normal steroid production. This study also demonstrated that PBR-depleted cells failed to produce pregnenolone when treated with exogenous cholesterol (Papadopoulos, 1997a). A role for PBR in the acute

stimulation of adrenocortical steroidogenesis has been implicated, possibly by mediating the entry, distribution and/or availability of cholesterol within the mitochondria (Papadopoulos, 1997b).

1.7.4.Steroidogenic acute regulatory protein

1.7.4.1 Introduction

The steroidogenic acute regulatory (StAR) protein was first described by Orme-Johnson and colleagues as an ACTH-induced, cycloheximide-sensitive, 30kDa phosphoprotein in hormone-treated rat adrenal (Krueger, 1983) and a luteinising hormone-induced protein in rat corpus luteum (Pon, 1986) and mouse Leydig cells (Epstein, 1991a). This protein, and an unphosphorylated form found mainly in quiescent cells, were found to be localised to the mitochondria (Epstein, 1991b). Similar mitochondrial proteins were reported by Elliot *et al* in bovine adrenal glomerulosa cells in response to ACTH and AngII (Elliott, 1993). Proteins similar to these were described in the MA-10 mouse Leydig tumor cell line and in addition to the 30kDa protein, a 37kDa precursor protein was also found (Stocco, 1988). The 30kDa protein, named StAR protein, was purified and the cDNA cloned and sequenced in MA-10 cells. A full length 1456-bp cDNA clone containing an open reading frame of 852 nucleotides encoding a protein of 284 amino acids (Clark, 1994). Full length cDNA clones for StAR have now been isolated for human (Sugawara, 1995a), bovine (Hartung, 1995), hamster (Fleury, 1996), rat (Lee, 1997) and pig (Pilon, 1997) and all have at least 84% homology. The StAR gene consists of seven exons split by eight introns (figure 1.14)

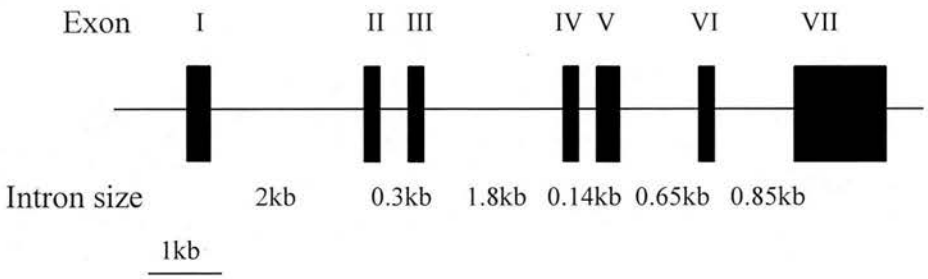

Figure 1.14 Schematic organization of the functional human/bovine StAR gene.

Transient transfection experiments demonstrated that that the expression of the cDNA-derived protein in MA-10 cells resulted in increased steroidogenesis in the absence of hormone stimulation (Clark, 1994). Similarly, when the non-steroidogenic COS-1 cell line was co-transfected with CYP11A, adrenodoxin, and StAR protein an increase in the conversion of cholesterol to pregnenolone was observed (Sugawara, 1995a). These results point to a role for StAR protein in hormone-regulated steroid production.

1.7.4.2 Lipoid congenital adrenal hyperplasia

Further evidence for a key function for StAR protein in steroidogenesis are the results from studies on congenital lipoid adrenal hyperplasia (lipoid CAH) (reviewed in (Miller, 1997). Lipoid CAH is a lethal condition that results from a complete inability of the infant to synthesis steroids. Affected infants die within days to weeks, due to the lack of mineralocorticoids and glucocorticoids unless treated with steroid hormone replacement therapy (Lin, 1995). As the mitochondria of affected adrenals and gonads fail to convert cholesterol to pregnenolone, the disease was thought to be the result of a defect in the CPY11A gene (Camacho, 1968), (Hauffa , 1985). The gene for CYP11A (Lin, 1991) and its protein levels (Sakai, 1994) were found to be normal in these patients. Other elements of the side-chain cleavage system,

adrenodoxin and adrenodoxin reductase, have also been shown to be normal (Lin, 1991). In addition, the genes and/or protein levels of the other proteins purported to play a role in the acute regulation of steroidogenesis, SAP, SCP2 and PBR were found to be normal in these patients (Lin, 1993).

Lin *et al* investigated the StAR gene for possible mutations and found nonsense mutations which resulted in the premature insertion of stop codons, thus producing truncated StAR protein. A number of other mutations have since been identified. Most patients are of Japanese or Korean ancestry, among whom the Q258X (mutation of glutamine to a stop codon at the 285th amino acid residue) mutation accounts for approximately 70% of cases (Yoo, 1998), [Katsumata, 1999), (Korsch, 1999), (Katsumata, 1998), (Bose, 1997), (Bose, 1998).

1.7.4.3 Expression of StAR

Studies using techniques such as western and northern analysis, in situ hydridisation, immunocytochemistry, RNAse protection assays and RT-PCR have shown that StAR expression is confined to steroidogenic tissues and the steroidogenic cells within these tissues (Clark, 1995b). StAR protein is not expressed in human placenta which implies that that this tissue has an alternative mechanism for the transfer of cholesterol to the side-chain cleavage system. This mechanism to date has not been elucidated (Clark, 1995b). However StAR protein has been found in the placenta of cow (Pescador, 1996), pig (Pilon, 1997), and rodent (Arensburg, 1999). StAR is expressed in both glomerulosa and fasciculata/reticularis cells of the adrenal cortex (Nicol, 1998), (Nishikawa, 1996), (Pollack, 1997), (Peters, 1998), (Cherradi, 1998a), (Hartung, 1995).

1.7.4.4 Transcriptional control of StAR

StAR expression is subject to both positive and negative regulation by various agents which presumably act on its promoter. In early studies it was shown that hormone-

stimulated steroid production was accompanied by a rapid rise in StAR mRNA (Clark, 1995b). Trophic hormone-stimulation usually results in an increase in intracellular cAMP. cAMP was found to have a positive effect in regulating StAR gene expression. Most studies have shown that the cAMP-responsive site was found within the first 254 nucleotides relative to the transcription start site. The StAR promoter however, lacks a full consensus cAMP response element. Therefore the cAMP response element binding protein may not act directly on sequences found in the StAR promoter (Sugawara, 1995b), (Caron, 1997), (Sugawara, 1997).

A transcription factor that maybe important in the regulation of the StAR gene is steroidogenic factor 1 (SF-1). SF-1 is a novel member of the orphan nucleotide receptor family of transcription factors that bind as a monomer to specific consensus response elements to induce the expression of the steroid hydoxylases (Morohashi, 1995). Several SF-1 consensus binding sites have been identified in the StAR promoter, with two of these sites (at positions -97 and -42) highly conserved in several species. Using transient transfection protocols, SF-1 has been shown to transactivate the StAR promoter in several cell types (Caron, 1997), (Sugawara, 1997), (Sandhoff, 1998), (Rust, 1998).

Additional factors are also involved in StAR's temporal and tissue-specific expression. The CCAAT/enhancer binding proteins (C/EBPs) are a family of basic region/leucine zipper transcription factors implicated as regulators of differentiation and function in multiple cell types (Johnson, 1993). Thus far six members of the C/EBP family have been identified. Two of these, C/EBPα and C/EBPβ, have been found in steroidogenic tissue and two putative C/EBP binding sites in the StAR promoter have been identified (Christenson, 1999), (Nalbant, 1998), (Reinhart, 1999), (Silverman, 1999). These studies have shown that the StAR promoter is transactivated by C/EBPβ during transient transfection assays. It has also been demonstrated that SF-1 and C/EBPβ interact as SF-1 transactivation of the StAR promoter has been shown to be dependent on the presence of C/EBP-binding sites

(Silverman, 1999). Sterol regulatory element binding protein 1a (SREBP-1a) may also be involved in the regulation of the StAR gene. Studies have shown the presence of potential SREBP sites in both the rat (Shea-Eaton, 2001) and human (Christenson, 2001) StAR promoters.

1.7.4.5 Models of StAR action

StAR was originally identified as a phosphoprotein, with the earlier work by Orme-Johnson demonstrating a strong correlation between the phosphorylated forms of StAR and steroidogenesis. The subsequent cloning of StAR cDNA from a number of species revealed that all of the known sequences contain two conserved consensus sequences for phosphorylation catalysed by protein kinase A, at two serine residues (S57 and S195) in humans and bovine (Arakane, 1997). Indeed, human StAR was shown to be rapidly phosphorylated on both these serine residues in response to 8-bromo-cAMP but only phosphorylation of S195 appeared to be functionally significant (Arakane, 1997). StAR protein has consensus sequences for phosphorylation catalysed by other kinases, including calcium/calmodulin protein kinase II and protein kinase C (Sugawara, 1995a).

The first model proposed that in response to stimulation by trophic hormones, StAR was rapidly synthesised in the cytosol as a 37kDa precursor and quickly targeted to the mitochondria via its signal sequence. As the transfer of StAR to the inner mitochondrial compartment begins, "contact sites" between the outer and inner membranes are formed. The formation of "contact sites" collapsed the intermitochondrial membrane aqueous space that served as a barrier to keep the hydrophobic cholesterol from crossing to the inner membrane, thus allowing cholesterol transfer via the newly formed lipid bridge. During mitochondrial import the signal and targeting sequences are sequentially removed by a matrix processing protease resulting in the 30kDa mature form of the protein. After processing of the 37kDa protein to the 30kDa, the membranes would separate and no further transport of cholesterol would occur. Therefore the continued synthesis and processing of StAR

precursor protein was required for the continued transport of cholesterol to the inner mitochondrial membrane (Stocco, 1996).

Several observations made this a viable model, firstly the fact that StAR was found to be localised to the mitochondria. Secondly the observation that the transport of mitochondrial proteins across the membranes occurs via "contact sites" (Pfanner, 1990). Finally it was demonstrated that mitochondrial "contact sites" in bovine adrenocortical cells contained the first two enzymes in the steroidogenic pathway, CYP11A and 3β-HSD (Cherradi, 1995).

It soon became clear that a revision of this model was required when it was shown that mitochondrial import was not essential to the function of StAR (Arakane, 1996). It was found that COS-1 cells transfected to express StAR proteins lacking the N-terminal 62 amino acids, and thus all of the signal sequence, supported steroidogenesis to the same extent as wild-type StAR, yet never entered the mitochondria (Arakane, 1996). Similarly, recombinant StAR lacking the N-terminal 62 amino acids could fully support steroidogenesis in isolated mitochondria without being imported into the mitochondria (Arakane, 1998). It seems therefore, that import of StAR is not required for cholesterol transfer to the inner mitochondrial membrane.

Truncations of the C-terminus, by 28 amino acids, resulted in the complete loss of steroid production (Arakane, 1996), indicating that the cholesterol transferring capability of the StAR protein resides in the C-terminal region (Lin, 1995), (Arakane, 1996), (Wang, 1998). This conclusion was reinforced by the finding that all mutations in LCAH are in the C-terminal region of the protein (Lin, 1995).

A study by Kallen *et al* demonstrated that StAR can also act as a sterol transfer protein and that the function of StAR may be to enhance desorption from one sterol-containing membrane to another (Kallen, 1998b). In this model, newly synthesised

StAR preprotein is selectively directed to the mitochondria by its N-terminal domain. On arrival at the outer mitochondrial membrane, the C-terminus acts on the outer membranes to produce alterations that favor cholesterol desorption to the inner membrane. Thus StAR's steroidogenic activity may be attributed to its ability to promote cholesterol movement from the relatively sterol-rich outer membrane to the sterol-poor inner membrane. The nature of the interaction between StAR and the outer mitochondrial membrane remains unclear (Kallen, 1998a).

Based on physico-chemical studies on recombinant StAR, Miller *et al* suggested that StAR acts on the outer mitochondrial membrane as a molten globule (Bose, 1999), (Bose, 2000), (Christensen, 2001). Most proteins are folded into unique conformations determined by energetic information specified by their sequences. Folding can proceed through a variety of intermediate states, comprising partially folded forms of individual domains or of the entire protein. Intermediates which have lost at least some of their tertiary structure but which have retained virtually all of their secondary structure are termed molten globules. Proteins may go through molten globule intermediate states during membrane insertion (Pilon, 1997). Subjecting StAR to limited proteolysis at different pH values it was demonstrated that StAR behaves differently as the pH decreases. StAR was shown to form a molten globule structure at pH 3.5-4.0, thus suggesting that StAR is biologically active at local acidic pH (Bose, 1999). Bose *et al* speculated that the acidic state of the outer mitochondrial membrane is caused by a transfer of protons across the two mitochondrial membranes. A proton pump produces an electrochemical gradient leading to a reduction in pH outside the mitochondria. StAR undergoes conformational changes that result in a partial unfolding of the protein and a transition to a molten globule state. Transition to a molten globule may lower the energy required to open the StAR structure further, possibly exposing a cholesterol channel or it may prolong the interval with which StAR can reside on the outer membrane, thus providing more time for the biologically active C-terminus to interact with the outer membrane (Bose, 1999).

Elucidation of the crystal structure of StAR protein has proven difficult. StAR protein is difficult to crystallise because it is not soluble to the extent required to form crystals. Therefore, information on the tertiary structure of StAR was not available until Tsujishita & Hurley crystallised and solved the structure of START domain of the MLN64 protein (Tsujishita, 2001). MLN64 is a 50 kDa protein of unknown function that is specifically expressed in the malignant cells of breast carcinomas. The C-terminus of MLN64 shares significant homology with StAR protein (Watari, 1997). The StAR related transfer (START) domains are 200-210 amino acid motifs that occur in a wide range of proteins, including the phosphatidylcholine transfer protein and acyl-CoA thioesterase, suggesting it has a role in lipid metabolism. The crystal structure of MLN64-START revealed an $\alpha+\beta$ fold built around a U-shaped incomplete β-barrel. The interior of the protein encompasses a hydrophobic tunnel that is large enough to bind a single cholesterol molecule. The START domain of MLN64 shows high homology (35%) with StAR-START domain and both can bind cholesterol in a ratio of 1:1. Based on the START domain structure and cholesterol binding stoichiometry, it was proposed that StAR functions in transferring cholesterol to the inner mitochondrial membrane via its ability to bind cholesterol and act as a cholesterol-shuttling protein (Tsujishita, 2001).

Mutations in the StAR gene have been shown to cause LCAH. When three of the most common mutations were mapped onto the MLN64-START domain model they were found to reside quite close to each other. Two of the of these mutations were found to reside within the cholesterol binding hydrophobic tunnel. Tsujishita and Hurley predicted that the LCAH-causing mutations would destabilise the tunnel resulting in a decrease in cholesterol binding (Tsujishita, 2001).

The role of StAR protein in cholesterol transfer remains a mystery. The fact that StAR does not need to be imported into the mitochondria to be fully active in

steroidogenesis does not agree with the proposal that StAR acts as a cholesterol-shuttling protein. Also at odds with this concept is the fact that once StAR is imported and processed from the 37 kDa precursor to the 30 kDa mature protein it is no longer active in cholesterol transfer, implying that StAR would not be able to act as a carrier protein on a continuous basis.

It has been postulated that StAR protein interacts with other mitochondrial outer membrane proteins and/or phospholipids to produce its effect. However attempts to identify such binding partners have produced no positive results. A study using fluorescence energy transfer demonstrated that StAR and PBR are closely associated on the outer mitochondrial membrane (West, 2001). Based on this close association West et al proposed that cytoplasmic StAR targets cholesterol to the mitochondrial membrane and that StAR then transfers cholesterol to PBR for import across the membrane space (West, 2001). This would be in keeping with work by Papadopoulous et al, who found that in the absence of PBR, StAR is incapable of stimulating steroidogenesis (Papadopoulos, 1997a).

Although PBR may play a role in cholesterol transfer, little is known about the mechanism of its action in this process. Moreover, the fact that StAR protein works in a non-steroidogenic host (COS-1 cells) indicates that StAR protein can transfer cholesterol into the mitochondria in the absence of outer mitochondrial membrane proteins, either as a sterol transfer protein, a molten globule or as cholesterol-shuttling protein (Kallen, 1998b), (Christensen, 2001), (Tsujishita, 2001).

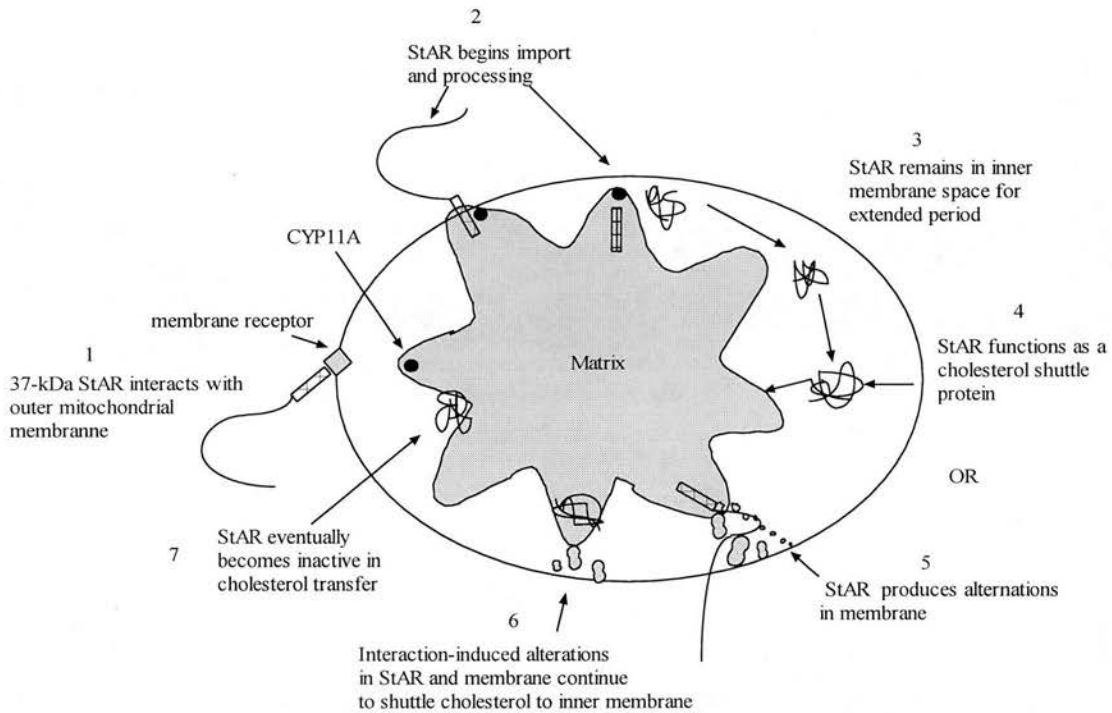

Figure 1.15 Putative mechanisms for StAR action. Shown are several ways in which StAR has been hypothesised to deliver cholesterol to the inner mitochondrial membrane. Upon trophic hormone stimulation, the 37-kDa protein precursor is synthesised and transported to the mitochondrial membrane. The 37-kDa protein interacts with a specific receptor protein on the outer mitochondrial membrane, at this time the insertion process begins. StAR itself may function as a cholesterol-shuttling protein. The hydrophobic regions in the START domain may alter the mitochondrial membrane and StAR structure to allow for the passage of cholesterol.

1.8 Adrenal cells in culture as a model for study

Many of the pioneering studies on adrenocortical function were performed using intact animals *in vivo* (Orth, 1992). The study of the adrenal cortex in its natural environment has certain advantages; however, the concentration of adrenal hormones in the plasma are very low and require sensitive assays. The measurement of steroid hormones in body fluids gives little information about the source of the hormones or to what extent they have been metabolised. The advent of *in situ* methods, where the adrenal vein is catheterised, allowed the measurement of adrenal hormones in the effluent blood (at a higher concentration than plasma), while maintaining the organ in its natural environment.

In vivo methods do not allow the researcher to distinguish between the cell types of the adrenal, nor can the effects of cortico-medullary interactions be analysed. They are also unsuitable preparations with which to study the cellular mechanisms of agonist stimulated steroidogenesis. Examination of the steroid capacity of adrenocortical cells began when the glands themselves or slices or parts were incubated at 37°C in buffered solutions (Vinson, 1978). Added substances, particularly potential precursors, gave valuable information about the steroidogenic pathway. The use of slices of adrenal tissue suffered from poor oxygenation and impaired delivery of agonist to the inner cell layers. Thus reducing the steroidogenic response when compared to *in vivo* or *in situ* studies cells (Kloppenborg, 1968). Such preparations also contained a variety of cell types and methods were sought for separation. The simplest of these was to remove the outer tissue capsule of the gland, thus stripping off the zona glomerulosa and the cells adhered to it (Vinson, 1978). This was refereed to as the capsular portion of the gland and the remaining zona fasciculata/reticularis was called the decapsulated gland.

The next advance was to groups or suspensions of one cell type. Adrenal tissues slices were digested using collagenase or trypsin (Kloppenborg, 1968), (O'Hare,

1973). The early studies using cultured rat adrenocortical cells were carried out on mixed populations of zona glomerulosa and fasciculata cells (Tait, 1974). Also early studies using bovine adrenocortical cells were either unpurified freshly isolated cells prepared from adrenocortical slices (Hepp, 1977) or unpurified cultured cells prepared from crudely dissected inner zone adrenal cortical cells (Gospodarowicz, 1977). Therefore interpretation of *in vitro* results with stimuli which affected both zones has been difficult. The need to use purified cell preparations was noted and several methods are now available for the purification of dispersed cells, these include gradient density centrifugation or column filtration (Tait, 1974), (McDougall, 1979). The cells of choice can then be maintained in a chemically defined media. Isolated cells offer clear advantages in that there is free access of the test substances to the receptor on the cell membrane and the steroids accumulate in the medium overlying the cells at a high concentration (Vinson, 1978). Also they allow a large number of incubations to be carried out with identical amounts of tissue from a small number of animals.

Various researchers have shown that ACTH stimulates glucocorticoid synthesis in adrenocortical cells at physiological doses which are lower than those required when tissue slices are used (Kloppenborg, 1968). Further, ACTH log-dose response curves for glucocorticoid output have been constructed and this output has been correlated with cAMP production (Hanning, 1970), (Lowry, 1973), (Beall, 1972). Gill *et al* postulated that raised cytosolic levels of cAMP activated a protein kinase as the next step in the mechanism of ACTH action (Gill, 1970). Cultured cell preparations have been increasingly used to investigate the acute second messenger and steroid secretory responses to steroidogenic agonists and the longer term effects on steroidogenic enzyme activity. The ability to maintain homogeneously dispersed cells from rat or bovine adrenal cortex in primary culture has provided a valuable *in vitro* model system for the study of the mechanisms which maintain adrenocortical structure and regulate its function. (Williams, 1989).

1.9 Aims of the thesis

1) To investigate the mechanisms responsible for the kinetics of cortisol production of primary cultured BAC cells whereby the rate of cortisol secretion declines markedly between 6 and 12 hours of ACTH-treatment.

2) To investigate whether there is any relationship between the kinetics of cortisol secretion and the expression of StAR mRNA in BAC cells.

3) To investigate whether local feedback mechanisms for cortisol secretion are exhibited by primary cultures of BAC cells.

CHAPTER 2: MATERIALS & METHODS

2.1 MATERIALS

2.1.1 List of Chemicals

Commercial Source

ACTH (1-39)	Sigma
Agarose (electrophoresis grade)	Boehringer Mannheim
Allihn funnels	Merck
Amphotericin B	Life Technology
Ampicillin	Life Technology
Androstenedione	Sigma
11β-hydroxyAndrostenendione	Sigma
Angiotensin II (asp^{1}- Val^{5}; MRC standard 64 / 15)	NIBSC
Anti-cortisol	SAPU
Bactotrytone	Life Technology
Bovine serum albumin (fraction 5)	Sigma
Bovine serum albumin (nuclease free)	Life Technology
Bromophenol blue	Sigma
10x Buffer B	Boehringer Mannheim
C18 μBondapak chromatograhy column	Waters
Cellulose wadding	Euroso Ltd
Chloroform	Merck
Collagenase (type 1)	Worthington
cortisol-3-CMO-2(^{125}I)-iodohistamine	Amersham

Corticosterone	Sigma
Cortisone	Sigma
CPSR-1	Sigma
[α-^{32}P]-dCTP	Amersham
Cycloheximide	Sigma
DE18 filters	Whatman
DEAE Sephedex A25	Sigma
Detergent (7X-PF)	ICN
Deoxycorticosterone	Sigma
Deoxycortisol	Sigma
Dichloromethane	Merck
Diethyl pyrocarbonate	Sigma
Donkey anti-sheep	SAPU
Dulbecco's modified Eagle medium/F12 (DMEM/F12)	Life Technologies
Duralon nylon membrane	Stratagene
Earle's balanced salts solution (EBS)	Life Technologies
EDTA	Boehringer Mannheim
Ethidium bromide	Sigma
Ethanol, absolute	Merck
Forskolin	Sigma
Glyoxal	Sigma
Guanidinium thiocyanate	Sigma
Ham's F-10	Life Technologies
Hydrogen peroxide (30% v/v)	Sigma
Hydrocortisone	Sigma

Immulite® ACTH kit	DPC
Isopropanol.	Merck
Klenow enzyme	Boehringer Mannheim
Kodax XAR-5 film	Sigma
L-broth	Life Technologies
Methanol (HPLC grade)	Merck
Microfuge tubes	Sarstedt
NAP 5 columns.	Pharmacia
Neubauer Haemocytometer	Mackay & Lynn
Normal sheep serum,	SAPU
Nylon gauzes (30, 100, 250 μm)	Lockertex
Penicillin/streptomycin	Life Technologies
Plastic containers, 30, 50, 250ml sterile	Mackay & Lynn
Polypropylene RIA tubes	Sarstedt
Phenol.	Amersham
QIAGEN Plasmid Maxi kit	Qiagen
Qiaquick kit	Qiagen
QuikHyb Hybridization solution.	Stratagene
RNA molecular markers	Life Technologies
Restriction enzymes (*Pst* I, *Eco*RI, *Hin*dIII)	Boehringer Mannheim
Salmon sperm DNA	Sigma
Sephadex G-10 and G-50	Sigma
Synacthen 250μg (ACTH 1-24)	Ciba

Tissue culture plates	MacKay & Lynn
Whatman 3MM filter paper	MacKay & Lynn
Whatman grade 1 filter paper	MacKay & Lynn
Yeast extract	Life Technologies

All chemicals were AnalaR grade and obtained from Sigma or Merck, unless stated otherwise,

2.1.2 Company Addresses

Amersham International PLC, Little Chalfont Buckinghamshire, UK

BDH Merck, Lutterworth, Leicestershire, UK

Biddy Sterilin, Stone, Staffordshire, UK.

Boehringer Mannheim UK, Lewes, East Sussex, UK

Ciba Laboratories, Horsham, West Sussex, UK

Diagnostic Products Corporation (EURO/DPC), Gwynedd, UK

Euroso Ltd, Whitestone Business Park, Whitestone , Hereford, UK

ICN Pharmaceuticals Ltd, Basingstoke, Hampshire, UK

Life Technologies, Inchinnan Business Park, Renfrewshire, UK

Lockertex, Warrington, UK

MacKay & Lynn, Edinburgh, UK

NIBSC, Potter's Bar, Hertfordshire, UK

Pharmacia Biotech Ltd Milton Keynes, UK

Qiagen Ltd, Dorking, Surrey, UK

Sarstedt, Leicester, UK

Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire, UK

Sigma, Poole, Dorset, UK

Stratagene Ltd, Cambridge, UK

Waters, Milford, UK

Worthington, Lorne Laboratories Ltd, Twyford, Berkshire, UK

Non-commercial source :

The StAR plasmid was a generous gift from Dr. Richard Ivell, Institute for Hormone and Fertility Research, Hamburg, Germany.

Etomidate, trilostane and ketoconazole were kindly supplied by Professor Ian Mason.

2.2 METHODS

2.2.1 Bovine adrenocortical cell preparation

Bovine adrenal glands were obtained from the local abattoir. The glands were trimmed of fat and 100μm slices were obtained using a Stadie-Riggs microtome. The first slice, consisting of the capsule and the zona glomerulosa, was discarded and the second slice, the zonae fasiculata/reticularis (zfr), was placed into a pre-weighed pot containing Earle's balanced salt solution (EBSS)/0.2% (w/v) bovine serum albumin (BSA). Slices showing visible medullary tissue were discarded.

Digestion method 1

5g of tissue was collected, chopped with scissors into 1-2mm fragments, washed with EBSS/BSA and digested with 50ml of collagenase (type 1) 2mg/ml at 37^{o}C for 2 hours, with shaking every 30 minutes. The resultant cell suspension was then filtered, by gravity, using 250 and 100μm mesh nylon gauzes to remove undigested tissue and then centrifuged for 20 minutes at 450g to pellet the cells. The supernatant was discarded and the cell pellet resuspended in EBSS/BSA and filtered through a 30μm gauze to give a single cell suspension. The cell suspension was then purified using a column filtration method [McDougall, 1979) which involves the cells being drawn, by gentle suction, through a sintered glass Allihn funnel (100mm x 20mm disc: 16-40μM pore size) containing 1ml Sephadex G-50 layered on top of 3ml of Sephadex G-10, previously equilibrated with EBSS/BSA. The G-50 serves as a course filter to remove any remaining larger debris whilst the G-10 selectively allows passage of red blood cells, zona glomerulosa cells and finer cell debris through the column, retaining the larger zfr cells in the interstites of the Sephadex G-10 gel.

The cells were recovered by resuspending the Sephadex gel in 50ml of EBSS/BSA and filtering through another 30μm gauze trapping, the beads but allowing the zfr

cells to pass through. The resultant cell suspension was centrifuged for 30 minutes at 450g (Williams, 1989).

Digestion method 2

3g of tissue was collected and the slices rinsed with warmed (37°C) DMEM/F12 containing 1% penicillin/streptomycin (P/S) and amphotericin B. The slices were placed into a glass digestion pot with fresh DMEM/F12. The digestion pot was placed on a stirrer and incubated at 37°C for 20 minutes. The DMEM/F12 was removed and replaced with 20ml DMEM/F12 containing 0.25% trypsin. The slices were stirred at 37°C and the solution decanted after 15 minutes, this was repeated once more to ensure removal of any serum proteins which may inhibit the action of trypsin. Another 20mls was added to the slices and placed on the stirrer at 37°C for 20-30 minutes. The solution begins to appear cloudy with cells and debris. The solution is decanted into a 250ml pot and retained. The remaining tissue was broken down by up and down action of a 5/10ml pipette, fresh digestion mixture was added and incubated for a further 20 minutes on the stirrer at 37°C. This process was repeated until only the connective tissue was left. The cell suspension was filtered with a 100μM filter and centrifuged for 30 minutes at 450g.

For both digestion methods the pelleted cells were resuspended in 50ml of growth medium (10% serum in the form of controlled processed serum replacement-1 (CPSR-1) in Ham's F-10 supplemented with 100 IU/ml penicillin/streptomycin (P/S) and 25μg/ml amphotericin B (Amp B). The cell yield was determined by counting on a haemocytometer, plated into culture dishes and maintained in an incubator at 37°C with a humidified atmosphere of 95% air, 5% CO_2

The trypsin digestion method was a less labour intensify alternative to the collagenase digestion. The two digestion methods produced comparable results when the isolated cells were treated with ACTH.

2.2.2. Treatment of cells with agonist and inhibitors

Cells were plated out in 25cm^2 tissue culture flasks at a density of 5x10^6 cells/flask, 12-well (0.33x10^6 cells/well) or 6-well (1.5x10^6 cells/well) culture plates. Unless stated otherwise the experiments were carried out on day 3 of culture. The cells were serum-deprived overnight in Ham's F-10 supplemented with 0.2% BSA plus P/S and Amp B and maintained in an incubator at 37°C with a humidified atmosphere of 95% air: 5% CO_2. The following morning the cells were washed twice with EBSS, then incubated with the appropriate agent, diluted in Ham's F-10 plus 0.2% BSA, P/S and Amp B, for the required period of time. The overlying medium was removed and stored at -20°C prior to steroid analysis. The cells were washed twice with EBSS and used for RNA or protein analysis. RNA extraction was carried out immediately. If this was not possible the cells were snap frozen with liquid nitrogen and stored at -80°C.

For the cells treated on various days in culture, the cells on day 0 were incubated in sterile 25cm universals, the cultured cells in 25cm^2 flasks. Before treatment with ACTH the cells were washed twice with Earle's balanced salts solution (EBSS). The medium changes on day 0 cells were carried out by centrifugation (450g for 30 minutes) to pellet the cells before resuspending in fresh medium. For cells in culture the medium overlying the cells was removed by aspiration prior to replacement by fresh medium. At the appointed time, termination of the experiment for day 0 cells was by centrifugation prior to removing the medium, and for the cells in culture the medium was simply removed from the flask.

2.2.3 Radioimmunoassay for cortisol

The assay consists of a double antibody preprecipitate to separate the bound and free phases. Using sheep anti-cortisol-3-carboxy-methyloxime (anti-cortisol-3CMO) antisera and a cortisol-3-CMO -^{125}I-iodohistamine [Gray, 1983 #2]. The preprecipitate was prepared from 650µl reconstituted sheep cortisol anti-serum, 530µl normal sheep serum, 15ml donkey anti-sheep and 10ml 0.1M citrate buffer (0.02% (w/v) gelatin, 0.013% (w/v) sodium azide), which was mixed and left overnight at 4°C. The solution was then centrifuged, at 230g for 15 minutes, the supernatant carefully decanted and the pellet resuspended in 500ml 0.1M citrate buffer.

Standards were prepared using cortisol (10mM dissolved in ethanol) in Ham's F-10/0.2% BSA, giving values of 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000nmol/L cortisol (Figure 2.1). Quality control samples representing 8, 80, 800 nmol/L cortisol were also prepared, to check for intra-assay drift and inter-assay precision. Inter- and Intra-assay variations were routinely found to be < 10%. The minimum detection limit was 7.1nmol/l. The sheep anti-cortisol-3-carboxy-methyloxime (anti-cortisol-3CMO) antisera is highly specific for cortisol, with the following cross-reactivity for other steroids: corticosterone 1.25%; progesterone 0.035%; 11-deoxycortisol 4.6%; deoxycorticosterone 1.92%.

Sample/standard/QC (100µl), 700µl of ^{125}I-cortisol (cortisol-3-CMO-(2-^{125}I) iodohistamine diluted in assay buffer (0.1M citrate; pH 4.0) to give ~ 10,000 cpm, and 250µl of the preprecipitate were added to polypropylene tubes, vortexed, and incubated for 70 minutes at 37°C. The samples were then centrifuged at 3000rpm for 30 minutes, the supernatant decanted by tipping the tubes onto cellulose wadding. The samples are then counted on a gamma-counter (LKB 1621 Multigamma), and the data analysed using RIA-calc software.

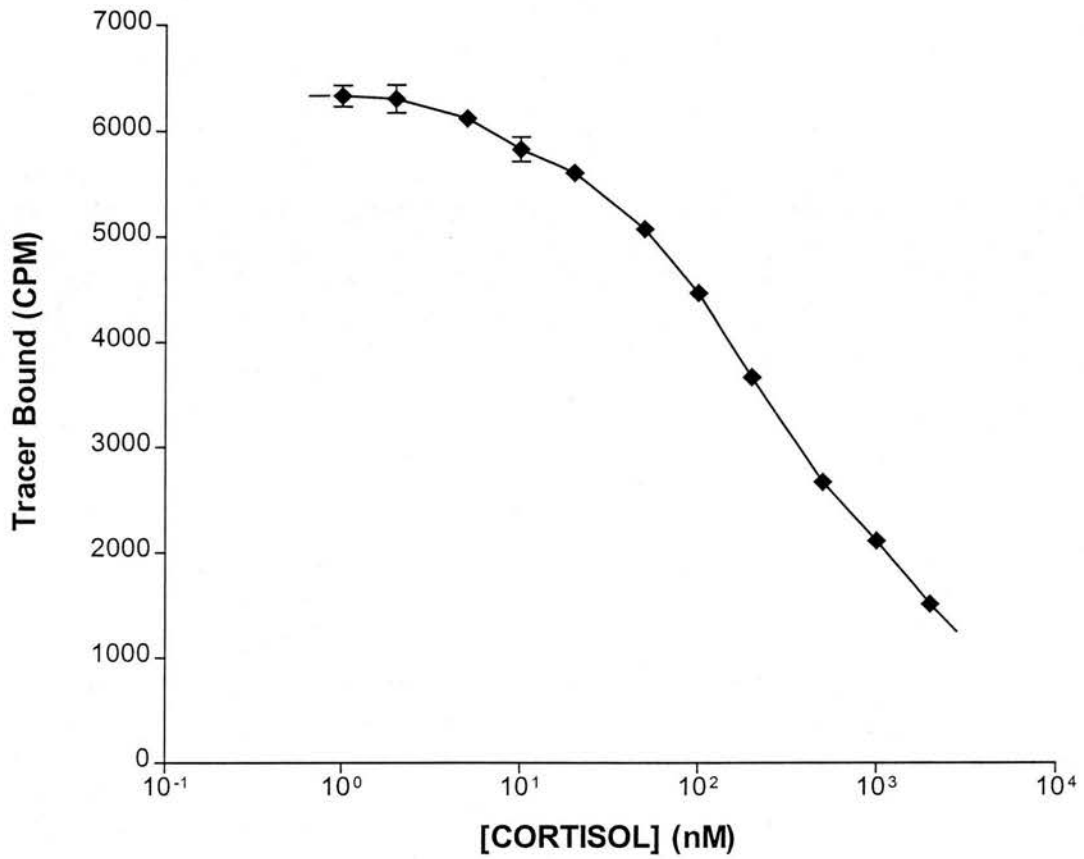

Figure 2.1 Representative standard curve for the ^{125}I radioimmunoassay for cortisol. Standard solutions for cortisol (1-2000nM, in Ham's F10 + 0.1% BSA) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of cortisol was typically 70 - 80% of the total tracer.

2.2.3 High Performance-Liquid-Chromatography (HPLC) for steroids

The profile of UV-absorbing 4-en-3-ketosteroids secreted by BAC cells in culture was determine by reversed-phase high performance liquid chromatography (HPLC) on a Hewlett Packard LC1100.

2.2.3.1 Optimisation

Optimisation of the HPLC was carried out (with the assistance of Duncan Campbell, as part of a student project), to determine the best volume of dichloromethane for extraction, to ensure that the amount of steroid extracted was proportional to the original concentration and to ascertain the retention times for each of the steroids for identification.

A known cortisol concentration added to Ham's F10 and extracted using various volumes of dichloromethane. The results shown in the table below are the mean of three independent samples. In parenthesis percentage of value obtained by RIA.

RIA	HPLC			
Cortisol	Volume of Dichloromethane			
(nmol/l)	1:1	1:6	1:9	1:12
5	3.2 (61%)	3.1 (58%)	4.6 (87%)	4.9 (94%)
38	17.1 (45%)	24.6 (65%)	35 (94%)	34 (91%)
443	256 (58%)	204 (46%)	335 (76%)	378 (85%)

In order to be able to calculate how integrated peak area related to the amount of steroid injected, a mixture of seven steroids were injected (cortisone, cortisol 11β-hydroxyandrostenedione, deoxycortisol, deoxycorticosterone, testosterone and 17α-hydroxyprogesterone). The stock mixture (1mg/ml of each steroid) was diluted

(figure 2.2), to confirm that the peak area, adjusted for molecular weight, was directly proportional to the concentration of steroid in the sample injected.

Routine identification of steroids was based on their retention times. To determine the retention time for each of the steroids of interest, a solution, 0.1mg/ml in ethanol, of each steroid was injected on to the HPLC and the time of elution was noted. The retention times are shown in table 2.1.

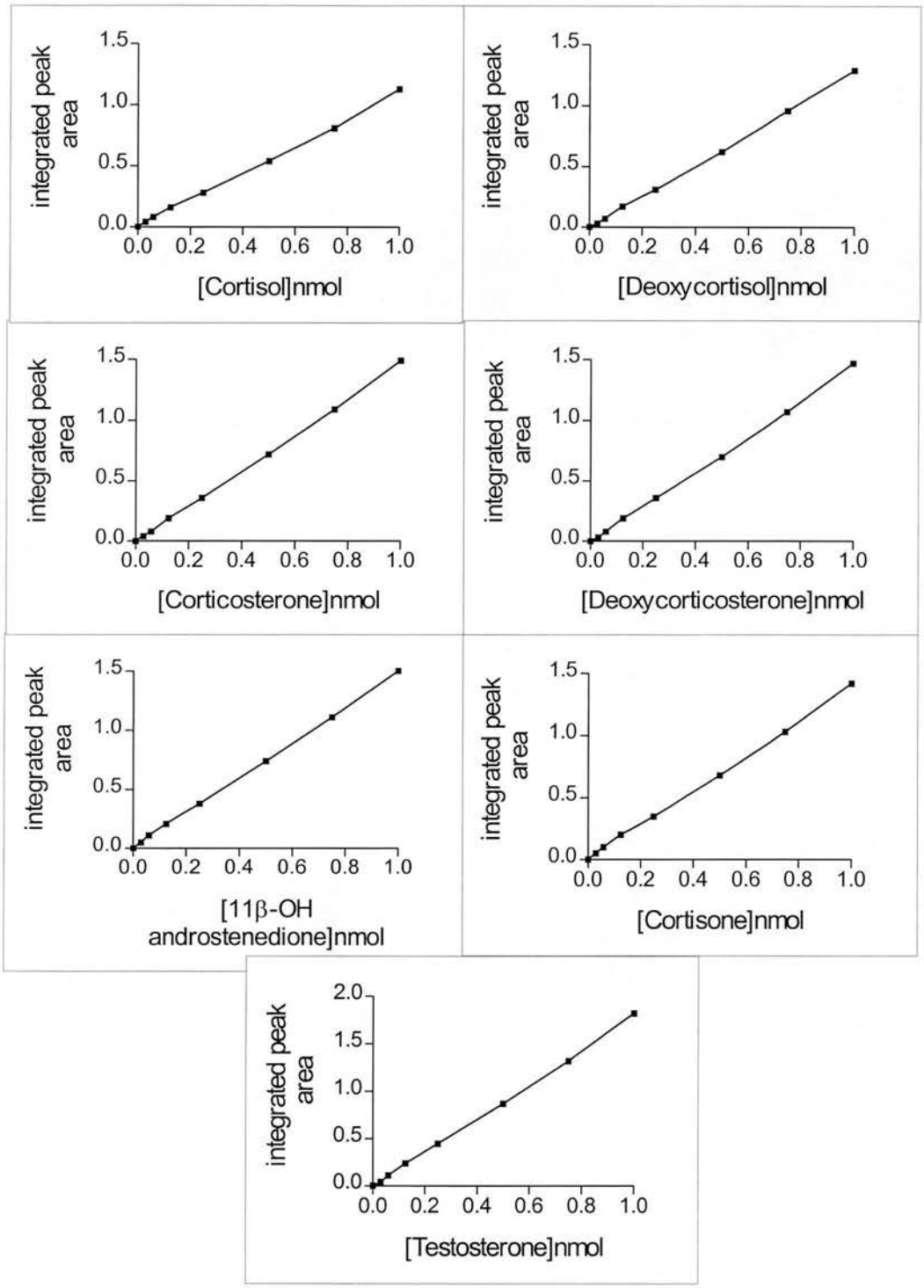

Figure 2.2 Integrated peak area relative to steroid concentration. Samples of known concentrations were injected onto the HPLC. The peak area was then calculated to be directly proportional to the concentration of steroid in the original sample.

STEROID HORMONE	MOLECULAR WEIGHT	FORMULA	RETENTION TIMES (minutes) ± 5%
Cortisone	360.4	$C_{21}H_{30}O_5$	5.81
Cortisol	362.5	$C_{21}H_{30}O_5$	6.99
11β-OH Androstenendione	302.4	$C_{19}H_{26}O_3$	8.37
Corticosterone	346.5	$C_{21}H_{30}O_4$	11.41
Deoxycortisol	346.5	$C_{21}H_{30}O_4$	12.94
Androstenedione	286.4	$C_{19}H_{26}O_2$	16.77
Deoxycorticosterone	330.5	$C_{21}H_{30}O_3$	21.83
Testosterone	287.4	$C_{19}H_{27}O_2$	24.55

Table 2.1 Retention times of steroids separated by HPLC. Retention times were determined by injecting each steroid, 0.1mg/ml steroid dissolved in ethanol, on to HPLC.

2.2.3.2 Method

Steroids, in the medium overlying the cells, were extracted using 1ml of medium to 9ml dichloromethane. Testosterone was added to the medium prior to extraction as an internal standard, used to determine the amount of recovery. The mixture was vortex and then centrifuged for 15 minutes at 1000rpm, the upper aqueous layer was removed and the solvent layer dried under air. The extracted steroids were then dissolved in methanol prior to injection.

The steroids were separated on a C18μBondapak reverse phase column in an isocratic system using 1:1 methanol : distilled water at a flow rate of 1.3ml/min, giving a column run time of 37 minutes. The absorptivity at 245nm was used to monitor the elution of the steroids. The eluted steroids were identified by their retention time and quantified by integrated absorption peak areas which are directly proportional to the quantity of steroid injected. A standard mixture (0.1mg/ml) of the steroids of interest was injected ahead of any samples, a trace is shown in figure 2.3.

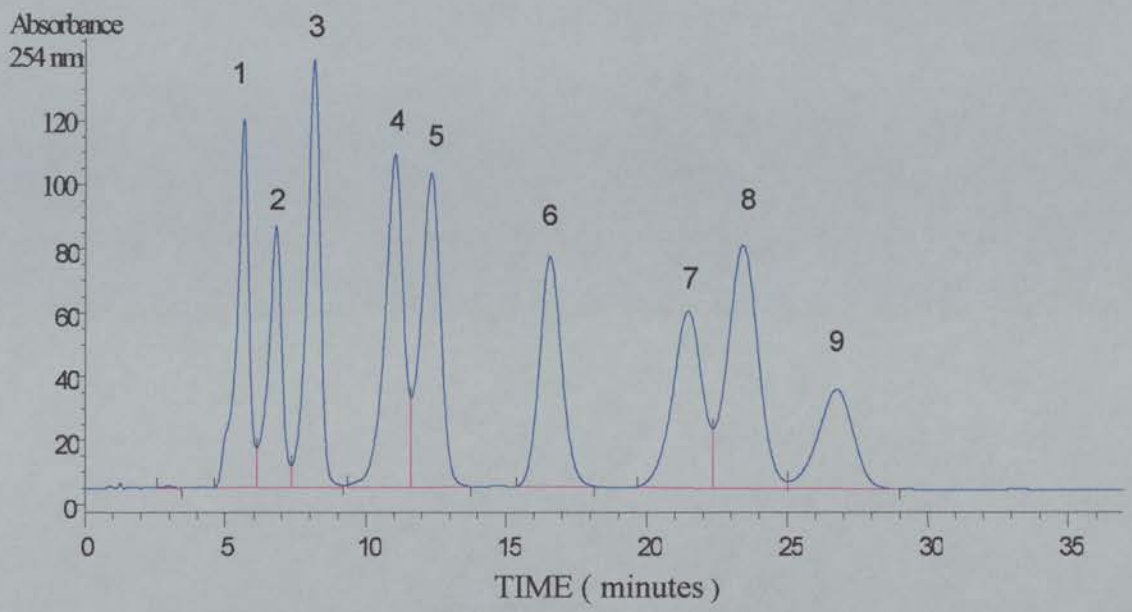

Figure 2.3. High performance liquid chromatograph of standard mix. Standard mix of UV absorbing steroids (0.1mg/ml). 1, cortisone; 2, cortisol; 3, 11β-hydroxyandrostenedione; 4, corticosterone; 5, deoxycortisol; 6, androstenedione; 7, deoxycorticosterone; 8, testosterone; 9, 17α-hydroxyprogesterone.

2.2.4 ACTH immunometric assay

ACTH was measured using a IMMULITE automated analyser (DPC), employing a solid-phase, two-site chemiluminescent enzyme immunometric assay. Polystyrene beads coated with a monoclonal, murine anti-ACTH antibody directed against the 18-39 C-terminal fragment and an alkaline phosphatase-labelled polyclonal rabbit anti-ACTH antibody directed against the 1-24 N-terminal fragment ensure that only the intact ACTH molecule is detected.

The sample and a buffer/serum matrix were simultaneously introduced into the test unit containing the labelled polystyrene beads and incubated for approximately 30 minutes at 37°C with intermittent agitation. ACTH in the sample binds to the antibody-coated bead, any unbound sample was removed by a centrifugal wash. The alkaline phosphatase-labelled antibody was introduced and the test unit was incubated for a further 30 minutes. The unbound enzyme conjugate was removed by a centrifugal wash.

The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, was added, and the test unit incubated for a further 10 minutes. The substrate undergoes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate. The continuous production of this intermediate results in the sustained emission of light. The bound complex was therefore proportional to the concentration of ACTH in the sample.

The IMMULITE system automatically calculates test results from the observed signal using a stored master curve. Calibration range is 9-1250pg/ml, intra- and inter-assay precision over the assay ranges was <10%. The ACTH antiserum was highly specific for ACTH, with low cross-reactivity to other ACTH fragments that maybe present in the sample.

2.2.5 Protein assay

The cells were washed twice with 1ml EBSS, 500µl of 1% Triton -X100 was added to each well and scrapped with the rubber end of a 1ml syringe plunger. The contents of the wells were then placed into a microfuge tube. The samples were diluted 1:10 with distilled water prior to analysis by the dye-binding assay of Bradford (1976). The assay was adapted for use on a Cobas Fara (Roche Diagnostics) centrifugal analyser. The dye, coomassie brilliant blue G-250 was prepared as described by Bradford [Bradford, 1976) and filtered through Whatman grade 1 filter paper before use. A standard curve was constructed using a range of BSA concentrations (0-100µg/ml) which were prepared in 0.1% Triton -X100.

2.2.6 Measurement of glucose

Glucose is measured using the hexokinase method [Bondar, 1974). Glucose is phosphorylated in the presence of hexokinase and ATP, producing glucose-6-phosphate. The glucose-6-phosphate reacts with NAD^+ in the presence of glucose-6-phosphate dehydrogenase to form gluconate-6-phosphate and NADH. The increase in absorbency, at 340nm, is proportional to the glucose concentration.

2.2.7 Preparation of StAR cDNA probe

The 400 bp segment of bovine StAR cDNA (figure 2.4) corresponding to bp 210-608 (Hartung, 1995), contained within a pMOS*Blue* vector, was mixed with competent DH5 (*E.coli* strain) cells.

2.2.7.1 Preparation of competent DH5 cells

The DH5 cells were made competent by inoculating 1ml of bacteria culture into 100ml L-broth supplemented with 10mM $MgSO_4$ and 0.2% (w/v) glucose. This was incubated at 37°C, 225rpm for 2 hours to give an A600 of 0.2-0.3 OD. The cells were then cooled to 4°C on ice and centrifuged for 5 minutes at 1000g at 4°C. The cell pellet was resuspended in 10ml of ice cold Miller transformation solution (L-broth containing 10% (w/v) PEG 8000, 5% (w/v) DMSO, 50 mM $MgCl_2$, pH 6.5), and either used directly for transformation or frozen in 200µl aliquots on ethanol:dry ice and stored at -70°C (Chung, 1989).

2.2.7.2 Transformation of bacteria with cloning vector

The tubes containing the pMOS*Blue* vector/competent cells mix were incubated for 30 minutes at 4°C. Pre-warmed SOC medium (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added (0.9ml) and the tubes incubated for 1 hour at 37°C, 225rpm, to allow expression of ampicillin resistance. The transformation mix was then plated, at various dilutions, onto agar plates, containing 100µg/ml ampicillin. The plates were incubated overnight at 37°C. Ampicillin-resistance colonies on the plate were then picked off and grown up overnight in either 100 or 400ml L-broth and ampicillin. Prior to using or storing the bacteria an aliquot of the L-broth culture was subjected to alkaline lysis miniprep to ascertain whether the plasmid inserted into the competent cells contained the required insert. The bacteria was then pelleted and frozen prior to further processing.

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gcagctgcag ggcccgaggc cacaaccctg tcaggctgag gaagcacagg agcagccatc    60
ctccgggacc agaggccaca gcaggagccc tcagcatccc cgccagactc cacacctgcc   120
cctactgcca ggaaagatgc tgctcgcgac gtttaagctg tgtgccggaa gctcctacag   180
acatgtgcgc agcatgaagg ggctgcagca gcaggctgtg ctggccatcg gccaggagct   240
gaaccggagg gccctagggg gcccggcccc agctgcgtgg attaaccagg ttcggcgtcg   300
cggctctctc ctaggttctc agctggaaga ccctctctac agcgaccaag agctggccca   360
tatccagcag ggagaggagg ccatgcagtg ggccctgggc atcctcaaag accaggaggg   420
ctggaagaag gagagccggc aggccaatgg ggacgaggtg ctgagtaaag tgatccctga   480
cgtgggcaag gtgttccggt tggaggtggt ggtggaccag cccatggaga ggctttatga   540
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catgtgtgtg ctggctggca tggccacact ctatgaggag atgccccagc agaagggtgt   780
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aaagatctca tctgaggaca gtgggacaag gtggtggcac gttttcaata agatactaca  1140
gctcagctac tacagcagca ttttagtacc aagagaatgc ggacaaggct cttctaactt  1200
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attaatattt attgtttctt attagtctat ttttggcata gatgaaatta tgtttttcca  2460
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gttttgagaa caggctgaca gtgagaaatg ggctttcaac acacaccatg tttgcctttt  2640
ccaaactatg ctactgtgtc tttgaggggc ctggggagat cttttctcac ttgtttttca  2700
ttggaataaa atgagttgtc agtgaaaaaa aaaaaaaaaa                        2740
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Figure 2.4_ Complete nucleotide sequence of the bovine StAR gene transcript. Underlined is the 400 bp segment of bovine StAR cDNA corresponding to bp 210-608 used as a probe for the northern hybridisation performed in this thesis. (Hartung, 1995).

2.2.7.3 Alkaline lysis minipreps of DNA

The transformed culture (1.5ml) was added to an microfuge tube and centrifuged in a microfuge at 12000rpm for 20 seconds to pellet the cells. Cell pellets were resuspended in 100µl of lysis buffer (25mM Tris.HCl pH 8.0, 10mM EDTA, 10% (w/v) glucose, 2mg/ml lysozyme) and left at room temperature for 10 minutes . 200µl 0.2M NaOH/1% SDS solution was then added and the mixture vortexed gently, and then placed on ice for 5 minutes. 150µl 3M potassium acetate (pH 4.8) was added and the solution vortexed for 10 seconds to ensure complete mixing. The tube was placed on ice for a further 5 minutes. and centrifuged for 1 minute in a microfuge at 12000rpm to pellet the cell debris and chromosomal DNA. The supernatant was then transferred to a clean microfuge tube, plasmid DNA precipitated by the addition of 0.9ml isopropanol and stored at -20^{o}C for 15 minutes. The solution was centrifuged for 2 minutes at room temperature to pellet the plasmid DNA, which was then resolubilised in 40µl TE (10mM Tris pH 8.0, 1mM EDTA) [Birnboin, 1979 #8].

2.2.7.4 Purification of DNA

Qiagen kits were used for these procedures and the company's protocols followed. The plasmid purification protocol was based on a modified alkaline lysis procedure followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions. RNA, proteins and other cellular impurities are removed by a medium salt wash. Plasmid DNA is then eluted in a high salt buffer and concentrated and desalted by isopropanol precipitation.

The bacterial pellet was resuspended in buffer P1 (50 mM Tris, 10mM EDTA, 100µg/ml RNase A), ensuring that no cell clumps form. The lysis buffer (200mM NaOH, 1% SDS) was added, the suspension mixed by inversion and incubated at room temperature for 5 minutes. Chilled neutralisation buffer (3M potassium acetate

(pH 5.5) was added and mixed by inversion. The lysate was then poured into the QIAfilter cartridge and incubated for 10 minutes at room temperature. The plunger was inserted and the cell lysate filtered into a QIAGEN-tip which was equilibrated with buffer QBT (750mM NaCl, 50mM MOPS, (pH 7.0), 15% isopropanol, 0.15% Triton X-100).The QIAGEN-tip was then washed with buffer QC (1.0M NaCl, 50mM MOPS (pH 7.0), 15% isopropanol), and the DNA eluted with buffer QF (1.25M NaCl, 50mM Tris, Tris-Cl, (pH 8.5) 15% isopropanol).

The DNA was then precipitated with 0.7 volumes isopropanol and centrifuged at ≥ 15,000g for 30 minutes. The supernatant was removed and the DNA pellet washed in 70% ethanol. The resulting pellet was air dried for 5-10 minutes and dissolved in a suitable volume of TE buffer (10mM Tris/HCl; pH 8.0, 0.4M EDTA). The yield was then determined by UV spectrophotometry.

2.2.7.5 Restriction enzyme digestion

DNA was generally digested at a concentration of 0.1-0.5μg/ml, the enzyme concentration was never more than 10% of the final volume to avoid problems with excess glycerol interfering with digestion. The digests were always carried out in the buffer recommended by the manufacturer of the enzymes. At the end of the digestion 0.5μg DNA was checked on an agarose gel to monitor the progress of the digestion. When digestion was complete the reaction was stopped by the addition of 2.5μl 0.4M EDTA/50 μl of reaction and heat inactivated at 65°C for 10 minutes. The DNA was then precipitated with 125μl 6M ammonium acetate and 750μl isopropanol and centrifuged for 5 minutes at 12000rpm. The pellet was washed with 180ml of 70% ethanol, centrifuged as before and briefly air dried before reconstituting in TE buffer.

2.2.7.6 Isolation of DNA probe

This procedure was carried out using a Qiagen kit, following the company's protocols. The DNA was separated on a gel and the DNA fragment of interest, visualised by ethdium bromide and UV, was excised from the gel with a clean sharp

scalpel, placed into a pre-weighed microfuge tube, and the tube re-weighed. 3 volumes of buffer QX1 (composition unknown) was added to 1 volume of gel (100 mg ~100µl) and the mixture incubated for 10 minutes at 50°C. To help dissolve the gel the tube was mixed by gently flicking every 2-3 minutes during the incubation. Isopropanol (1 volume) was added, the solution mixed and placed into a QIAquick spin column in a collection tube and centrifuged for 1 minute. The flow through was discarded and the QIAquick column washed with buffer PE (composition unknown), and centrifuged for 1 minute. The DNA was then eluted with 50µl of 10mM Tris-HCL, pH 8.5 or with water, depending on the future use of the probe, by placing the elution buffer onto the centre of the column and centrifuging for 1 minute. The DNA was then stored at –20°C.

2.2.8 RNA isolation from cells in culture

Cells in tissue culture dishes were washed twice with EBSS to remove any traces of growth medium. If the RNA was not extracted immediately the cells were snap frozen, by adding liquid nitrogen to the flask, and stored at -80°C. The cells were then lysed by the addition of of RNAzol, 0.2ml/million cells in accordance with the manufacturers instructions. The RNAzol was gently pipetted over the cells five times to ensure solubilisation of the RNA and then transferred to a clean, RNAse-free microfuge tube and kept on ice. 100µl of chloroform was added and the tubes shaken vigorously for 15 seconds, then left on ice for 5 minutes. After centrifugation at 12000rpm for 15 minutes to separate the phases, the upper aqueous phase, containing the RNA, was transferred to a clean microfuge tube.

The RNA was then recovered by precipitating with an equal volume of isopropanol for 30 minutes at 4°C, centrifuging at 12000rpm for 15 minutes, followed by washing the pellet with 180µl of 70% ethanol, centrifuging as before and air drying on ice for 10 minutes. The RNA pellet was resuspended in 100µl of RNAse-free water. Quantification of the RNA was by UV spectrophotometery and confirmation

of the RNA quality was carried out by gel electrophoresis (figure 2.5). The samples were then analysed by northern hybridisation. The RNA samples can be stored at –70°C prior to analyses.

2.2.9 Northern hybridisation

Northern blotting analysis involves separating RNA by electrophoresis followed by permanent transfer to a nylon membrane and hybridisation with the specific probe of interest. Prior to sample preparation the gel equipment was washed with 7X-PF detergent, soaked in 3% (v/v) hydrogen peroxide for 15 minutes, then rinsed in RNAse-free water and drained.

The RNA samples (25µg), 18 and 28S markers (5µg of a sample), molecular weight RNA markers, and a negative control (RNA containing no detectable StAR mRNA) were precipitated by the addition of 0.1x volume 3M sodium acetate (pH 5.5) and 3x volumes absolute ethanol to the RNA, and incubated overnight at -20°C. The following morning the samples were centrifuged for 15 minutes at 12000rpm to pellet the RNA. The pellets were washed with 180µl of 70% ethanol, vortexed, centrifuged and air dried for 5 minutes. The pellet was then resuspended in 3.7µl of RNAse-free water and 12.3µl of glyoxal/phosphate buffer/DMSO mix was added, the samples were denatured at 50°C for 1 hour. The samples were then cooled on ice and 4µl of RNA loading buffer (10mM Na Phosphate; pH 7.0, 50% (v/v glycerol, 0.4% (w/v) bromophenol blue) was added. The samples were loaded onto a 1.2% agarose gel (agarose/10mM phosphate buffer/RNAse-free water) with a running buffer of 10mM phosphate buffer at 100V for approximately 4 hours. The buffer was circulated to avoid ionisation of the phosphate buffer which raises the pH causing the glyoxal to dissociate from the RNA.

The RNA was then transferred to the nylon membrane by capillary transfer. Using a suitable receptacle (a pyrex dish) a capillary bridge was formed by placing three pieces of Whatman 3MM filter paper (cut to the same width as the gel and approximately three times as long) onto a glass plate with some 20xSSC (3.0M NaCl, 0.3M Na citrate) removing any air bubbles between the layers by carefully rolling a glass pipette over the filter paper. The pyrex dish was filled with a litre of 20xSSC. The gel was then placed on top of the filter paper and air bubbles removed as before (the top left hand corner of the gel was cut off to aid orientation). A piece of nylon membrane, cut to the same size as the gel and wetted in 2xSSC, was then placed on top of the gel, removing any air bubbles. Nine pieces of Whatman 3MM paper (cut to the same size as the gel) were placed on top of the membrane; the first six were wetted in 2xSSC and the air bubbles removed as described and the remaining three placed on the top dry. The sides of the wick were covered with parafilm to ensure that the transfer only took place through the gel and glass pipettes were placed on two opposite sides of the set-up to support the transfer. Absorbent tissues was placed on top of the Whatman paper (approximately 9cm) and a smaller glass plate placed on top of these with a 0.5kg weight. The transfer was left overnight.

The following morning the set-up was disassembled and the site of origin marked on the nylon membrane and the top left hand corner cut off for orientation purposes. The membrane was baked at 80^{o}C for 30 minutes to de-glycoxylate the RNA. The RNA was then covalently bound to the membrane by exposing it to 240mJ UV light at 312nm in a Bio-rad crosslinker.

The first two lanes on the membrane, containing the molecular weight markers and 18S/28S markers, were cut off and stained for 5 minutes in methylene blue (0.04% (w/v) in 0.5M Na acetate; pH 5.5). Destaining in distilled water revealed nucleic

acids stained blue. If the correct pattern of staining was observed the blot was then ready to be hybridised (figure 2.5).

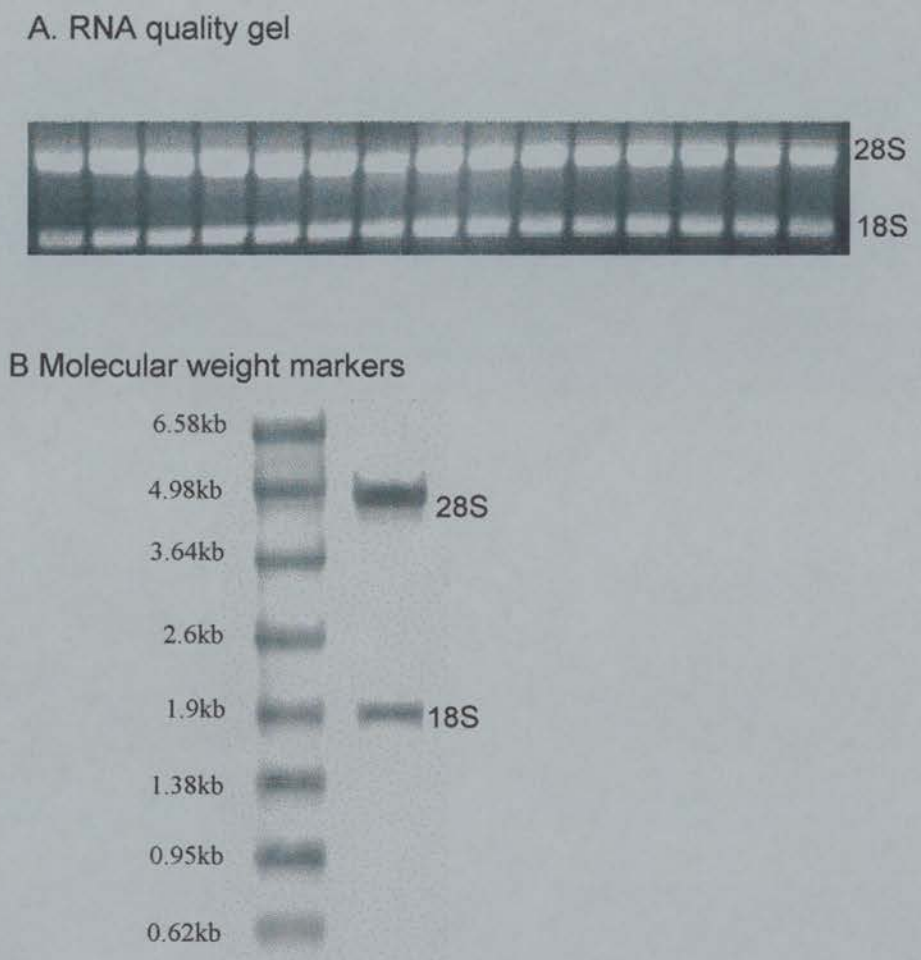


Figure 2.5 RNA integrity check gel and molecular weight markers . A: Gel run prior northern hybridisation to test quality of RNA. If gel shows clear bands with no smearing, as shown above, this indicates good quality RNA which is not degraded. B: RNA markers. Strip cut from northern hybridisation showing the molecular weight makers and 18 &28S RNA markers.

2.2.10 Hybridisation with Vogelstein random primer probe

To prepare the radiolabelled probe, 50ng of template DNA and 250ng of random primer were mixed with TE buffer (10µl volume), denatured for 5 minutes at 100°C followed by quenching on ice to avoid renaturation. The following were then added; 5µl of labelling buffer (0.5M Tris HCl; pH 6.9, 0.1M $MgSO_4$, 1mM DTT, 1mM dATP, 1mM dGTP and 1mM dTTP), 5µl [^{32}P]-dCTP (10µCi/µl), 26.5µl sterile water, 1µl nuclease free BSA (10 mg/ml) and 2.5µl Klenow enzyme (2 U/ml). The reagents were incubated for 1 hour at room temperature to allow incorporation of the [^{32}P]-dCTP into the template DNA (labelling).

After this time 1µl samples was removed to calculate the amount of radioactivity incorporated into the DNA probe. 0.5µl was spotted onto duplicate DE81 filter discs, dried and the radioactivity determined. The filters were washed in 0.5M Na_2HPO_4, rinsed with distilled water followed by ethanol and the radioactivity determined. The amount of radioactivity incorporated (% incorporation) and specific activity are then calculated.

The procedure was stopped by the addition of 2.5µl 0.4M EDTA pH 8.0 and the 'labelled' probe was then purified, removing 'unlabelled' nucleotides, by passing it through a NAP-5 column equilibrated with 6xSSC and eluting with 1ml 6xSSC. Probes used have a specific activity of 1-2x10^9cpm and were used at a concentration of ~1x10^6cpm/ml hybridisation solution.

QuikHyb was used as the hybridisation solution, according to the manufacture's instructions. The blot was soaked in 6xSSC for 5 minutes. The wetted blot was then placed into a Techne hybridisation bottle and hybridised with 10ml of Quickhyb solution for 10-20 minutes at 68°C. The Vogelstein random primer DNA probe was added to 100µl of salmon sperm DNA (100µg/ml) and both were denatured by

heating at 100°C for 10 minutes followed by cooling on ice. This solution was then added to the hybridisation solution and incubated for 3 hour at 68°C. The membrane was then washed twice for 15 minutes at room temperature with 2xSSC/0.1% SDS wash solution, followed by a 30 min wash at 60°C with 0.2xSSC/0.1% SDS wash solution. The membrane was briefly air dried, wrapped in saran-wrap and laid down on Kodak XAR-5 film with an intensifying screen at -70°C for 24-72 hours. For quantification analysis the membrane was laid on the BioRad phosphoimaging system and the hybridising bands and quantified using the BioRad imaging software.

The membranes were stripped by washing twice for 15 minutes in 0.1xSSC/0.1% SDS at 100°C and re-probed for the housekeeping gene, β-actin. This was to ensure equal loading of RNA in each well.

2.2.11 Statistical analysis

On the advice of Mr William Adams, Department of Medical Statistics, University of Edinburgh, the following statistical tests were used to analysis the data presented in this thesis. All data were analysed using Instat or Instatprism software.
Results are expressed as means ±SD. Comparison of means were assessed using Student's t-test and considered significant when $P<0.05$.
To assess the plateau in cortisol output an non-linear curve equation was fitted: $Y=Ymax*(1-exp(-K*X))$, where Y= cortisol value.
The peak in StAR transcript expression was extrapolated using a 2nd order polynomial quadratic equation: $Y=A+B*X+C*X^2$, where Y= StAR value.

CHAPTER 3 : CHARACTERISATION OF THE STEROIDOGENIC PROFILE TO ACTH BY BOVINE ADRENOCORTICAL (BAC) CELLS.

3.1 Introduction :

Adrenocorticotropin (ACTH) is a 39 amino acid polypeptide (MW 4500kDa) produced by the anterior pituitary from a precursor molecule called preopiomelanocortin (POMC), MW 28,500kDa (figure 3.1). Within the pituitary POMC is synthesised and proteolytically cleaved into several fragments including ACTH, β-lipotropin and β-endorphin (Orth, 1992). The primary action of ACTH is on the adrenal cortex where it increases glucocorticoid synthesis and secretion. The principal glucocorticoid secreted in humans and bovine species is cortisol. ACTH has much less effect on adrenal androgen production and, at physiological levels, virtually no effect on aldosterone production.

The biological function of ACTH is dependent upon the NH_2-terminal 24 amino acids, which are the same in all species studied. Any species variation occurs with the remaining 7 amino acids of the COOH-terminus. This part of the peptide forms a 'tail' that apparently stabilises the molecule within the body (the half-life of ACTH in circulation of humans is about 10 minutes) (Schwyzer, 1977). Replacement of glutamate, in the human sequence, at amino acid 33 by glutamine is the only difference in the ACTH sequence between human and bovine species (figure 3.1) (Schwyzer, 1977).

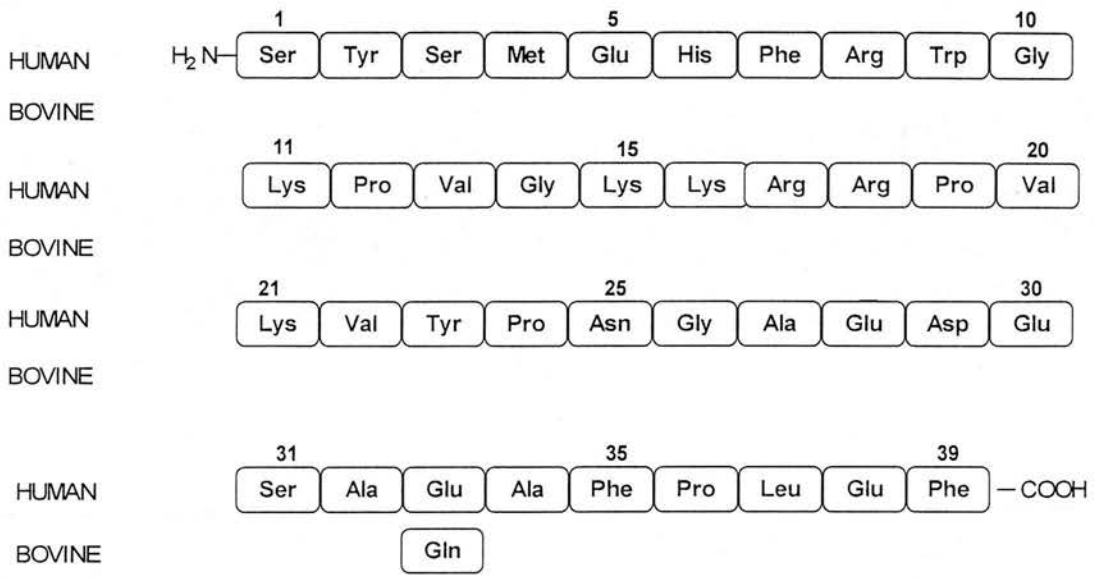


Figure 3.1 Amino acid sequence of ACTH. Modified from ACTH and related peptides: structure, regulation and action. (Schwyzer, 1977).

ACTH acts by binding to specific G-protein coupled receptors on the cell surface and activating intracellular signal transduction systems. The actions of ACTH are mediated via the cAMP second messenger system, as discussed in chapter 1.

It was once thought that ACTH was the sole stimulus of steroid hormones from the adrenal zona fasciculata/reticularis cells; however, it has now been shown that these cells respond to a wide variety of hormones and neurotransmitters, such as vasopressin (Walker, 1991b), AngII (Canny, 1998), acetylcholine [Walker, 1991a), (Clyne, 1993b), phorbol ester (Kenyon, 1988) and interferons (Tachikawa, 1999). ACTH acts via the adenylate cyclase second messenger pathway system to promote steroidogenesis in the adrenal cortex. This pathway can also be stimulated by the diterpene compound forskolin and 8-bromo-cAMP (8-Br-cAMP), a cAMP analogue. Forskolin activates adenylate cyclase in mammalian cells. However its actions are not mediated through interactions with any of the major classes of

receptors. In most, but not all, situations the actions of forskolin appear to be consistent with a direct action on the catalytic unit of adenylate cyclase (Seamon, 1983). Adenylate cyclase has been shown to have two forskolin-binding sites, a low affinity site being associated with the catalyst and a high affinity site being attributed to the activated complex of the catalyst (Seamon, 1984), (Nelson, 1986). Forskolin has been reported to stimulate cAMP and steroid hormone production in bovine adrenocortical cells (Langlois, 1987), (Yanagibashi, 1989), (Juska, 1995).

Other agonists, such as angiotensin II (AngII), do not have any effect on cAMP levels but exert their effects through the activation of phosphoinositidase C (PLC) (Bird, 1989). Much work has been done on the effect of AngII on aldosterone synthesis in the adrenal zona glomerulosa (Kojima, 1985a), (Farese, 1984), (Kojima, 1985b). Nevertheless the hydrolysis of phosphoinositides and the production of cortisol in BAC cells in response to AngII has been demonstrated (Bird, 1992b), (Clyne, 1992).

Bovine adrenocortical (BAC) cells have been used to study the mechanism of action of a variety of steroidogenic agents on different aspects of the steroidogenic response. BAC cells have be used individually (Kenyon, 1988), after immediate isolation (Walker, 1991b), (Nishikawa, 1996), (Yaguchi, 1998) or following culture (Le Roy, 2000), (Tachikawa, 1999), (Clyne, 1993b). The use of cells in culture is the more widely used approach but the length of time in culture before experimentation varies from 24 hours to 6 days after isolation.

The aims of this chapter were firstly, to try and determine the ideal culture conditions with which to study the steroidogenic response of BAC cells. The response of BAC cells on various days in culture, and the use of serum in the experimental culture medium were investigated. Secondly the steroidogenic response of BAC cells to ACTH over a 12 hour period was studied. This included

an identification of the various steroids produced by the BAC cells in response to ACTH-treatment, the response to various ACTH concentrations and the response to both synacthen ($ACTH_{1-24}$) and the native $ACTH_{1-39}$ peptide. Finally a comparison between the BAC cell response produced to ACTH and that produced by other agonists was studied.

3.2 Results

3.2.1 The responsiveness of BAC cells to $ACTH_{1-24}$ treatment at various stages in culture.

BAC cells were exposed (6 & 24 hour treatments) to $ACTH_{1-24}$ on the day of isolation (day 0) and on days 1, 2, 3, and 4 of culture. The cells on day 0 were placed in sterile 25 ml universals ($5x10^6$ cells/universal) and those on days 1, 2, 3 and 4 were cultured in 25 cm^2 tissue culture flasks ($5x10^6$ cells/flask). The cells were maintained in Ham's F10 medium containing 10% CPSR1 (a controlled processed bovine serum replacement used in place of serum) throughout these experiments.

Figure 3.2 demonstrates that, on each of the days studied, the amount of cortisol secreted into the medium overlying the $ACTH_{1-24}$-treated BAC cells was significantly increased compared with equivalent untreated cells at 6 hours ($P<0.05$) and at 24 hours ($P<0.05$).

There was a significant difference in the amount of cortisol production by BAC cells at 6 hours compared with 24 hour on each of the days apart from day 1. On day 0 there was a significant decrease in the cortisol output at 24 hours compared with 6 hour ACTH-treatment, $P<0.05$. On days 2, 3 and 4 the cortisol output in response to ACTH-treatment increased between 6 and 24 hours, $P<0.05$.

The cortisol output from the $ACTH_{1-24}$-treated cells at 6 hours was increased slightly over the different days in culture. A significant increase in the amount of cortisol produced by the cells was found on day 2 when compared with day 0 ($P<0.05$) and day 1 ($P<0.05$). No difference was found between the cells which had been cultured for 2, 3 or 4 days. $ACTH_{1-24}$-treatment for 24 hours also produced an increase in

cortisol output with time in culture. No significant difference was found in the cortisol output between days 0 and 1 at 24 hour ACTH-treatment. However there was a significant increase in the amount of cortisol secreted into the overlying medium after 24 hours ACTH-treatment on days 2, 3 and 4 when compared with the cortisol produced by BAC cells on day 0 ($P<0.001$). There was also a significant increase in the amount of cortisol secreted by BAC cells in response to 24 hour ACTH-treatment on day 4 compared with day 1 of culture ($P<0.05$). There was no significant difference ($P>0.05$) between the cortisol output at 24 hour $ACTH_{1-24}$ treatment on days 2, 3 and 4 of culture.

Based on these observations subsequent experiments were carried out on day 3, after an initial culture period of 2 days.

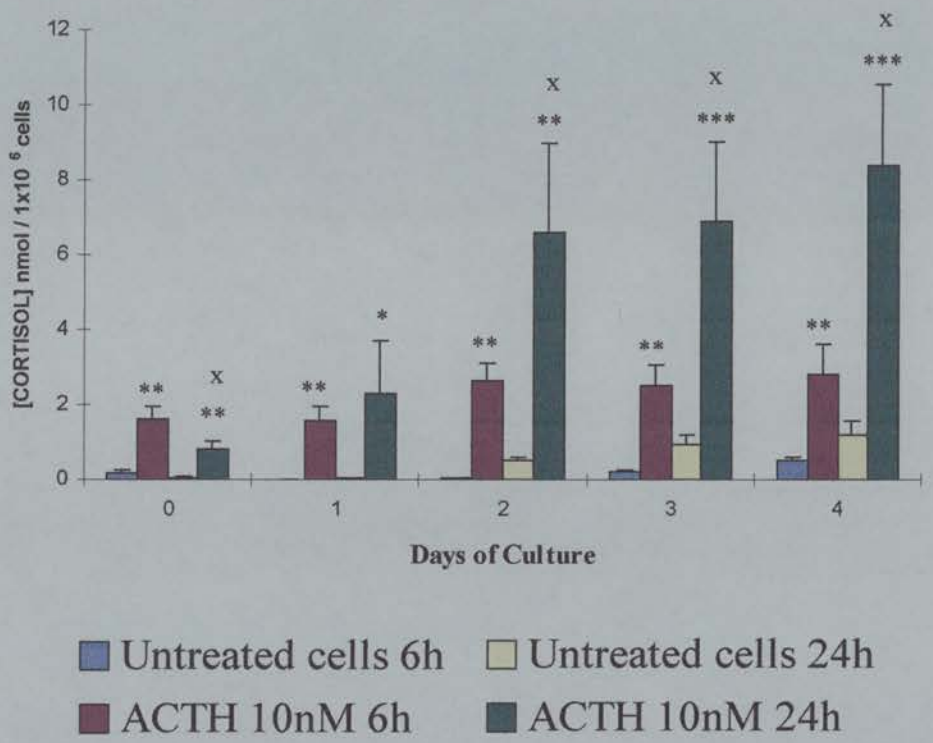


Figure 3.2 : Cortisol output by BAC cells in response to $ACTH_{1-24}$ treatment on separate days of culture. BAC cells were maintained in Ham's F10 + 10% serum throughout the experiment. The cells were treated with or without 10nM $ACTH_{1-24}$ as indicated. Results shown are mean ± SD of three independent cell isolations. * $P< 0.05$, ** $P<0.01$, ***$P<0.001$ comparing untreated cells with treated cells at each of the time points, on each day e.g. 6h untreated on day 0 v 6h ACTH treated on day 0 etc. x $P<0.05$ comparing 6h treated with 24 h treated on same day of culture

The comparisons between the 6h $ACTH_{1-24}$ treated cells and 24 hours $ACTH_{1-24}$ treated cells on separate days are discussed in the text.

3.2.2 Effects of serum on the cortisol response of BAC cells treated with $ACTH_{1-24}$

BAC cells were cultured for 2 days in $25cm^2$ flasks. One group of cells was maintained in Ham's F10 containing 10 % serum and a second group of cells was serum-deprived overnight prior to the experiment, i.e. the medium was removed and replaced with Ham's F10 + 0.2% bovine serum albumin (BSA). The following morning both sets of cells were washed twice with EBSS prior to $ACTH_{1-24}$-treatment in either Ham's F10 containing 10 % serum or Ham's F10 containing 0.2% bovine serum albumin (BSA). At the designated times the medium was removed and assayed for cortisol. Untreated cells were studied at 6 and 48 hours only.

The effect of serum on the pattern of cortisol production over a 48 hour period is illustrated in figure 3.3. In both the presence and absence of serum there was a significant increase in the amount of cortisol produced by $ACTH_{1-24}$-treated cells compared with untreated cells at 6 and 48 hours ($P<0.05$).

No significant difference was found in the amount of cortisol secreted into the medium overlying the $ACTH_{1-24}$-treated cells when maintained in either the presence or absence of serum.

From these results it is clear that 10nM $ACTH_{1-24}$ was adequate to stimulate BAC cells in the presence or absence of serum. Based on these findings all subsequent work with BAC cells was conducted in the absence of serum, unless stated otherwise.

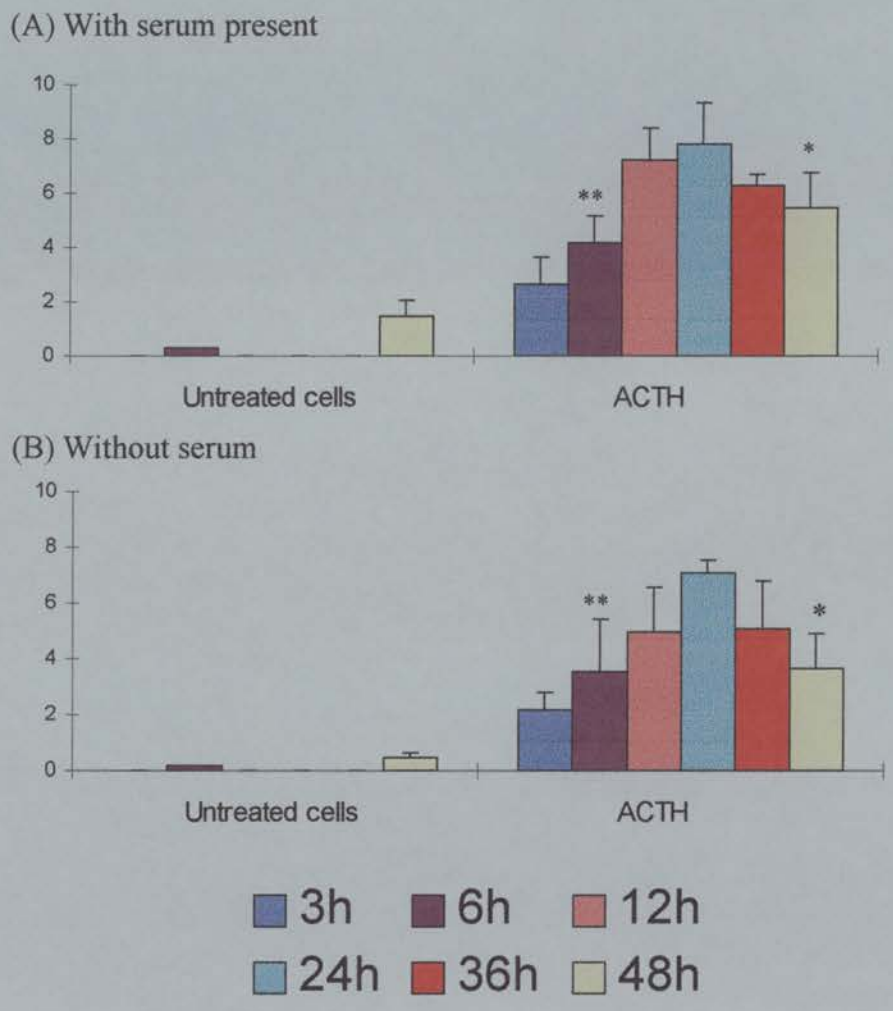


Figure 3.3 : Effect of serum on the cortisol output by BAC cells in response to $ACTH_{1-24}$ treatment. BAC cells were cultured in 25cm^2 flasks for 2 days, then either A, maintained in the presence of serum or B, serum-deprived overnight. The cells were treated with $ACTH_{1-24}$ (10nM) as indicated, untreated cells only at 6 and 48 hours. The medium overlying the cells was then removed and assayed for cortisol. Results shown are mean ± SD of three independent cell isolations. **P<0.05, **P<0.01 comparing 6 and 48 h untreated with 6 and 48h ACTH-treated cells.

3.2.3 Treatment of BAC cells with $ACTH_{1-24}$ over a 12 hour period

Exposure of BAC cells to $ACTH_{1-24}$ (10nM) resulted in a significant secretion of cortisol into the medium overlying the cells. Figure 3.4A displays means ± SD for four experiments each from independent cell isolations and figure 3.4B the combined means ± SD of the four experiments.

The results from each of the four independent experiments shown in figure 3.4A conform to a reproducible pattern of cortisol output. At each of the time points studied (1-12h) there was a significant increase in the cortisol concentration in the medium overlying the $ACTH_{1-24}$-treated cells compared with untreated cells at the same time point. Over the time course there was an increase in the rate of cortisol production by BAC cells over the initial four hours with a fall in the rate of production thereafter. Fitting a nonlinear curve equation the plateau in cortisol production was found to vary between 4 and 8 hours for the four experiments.

This pattern is reflected in the graph combining the four experiments (figure 3.4B). $ACTH_{1-24}$-treated cells displayed a significant increase in the amount of cortisol secreted into the medium overlying the BAC cells compared to untreated cells at each of the time points studied, $P<0.00$ at 1 and 2 hours, $P<0.001$ at 4, 6, 8 and 12 hours. Using a nonlinear curve fit equation it was found that the plateau occurred at a concentration of 3.2 ± 0.1nmol cortisol/10^6 cells, corresponding to the amount of cortisol secreted into the medium after 6 hours of $ACTH_{1-24}$-treatment.

Figure 3.5 displays the protein content of BAC cells over the course of the experiment. This did not vary by more than 10% over the 12 hour time period, thus indicating no significant decrease in the number of cells in the culture wells.

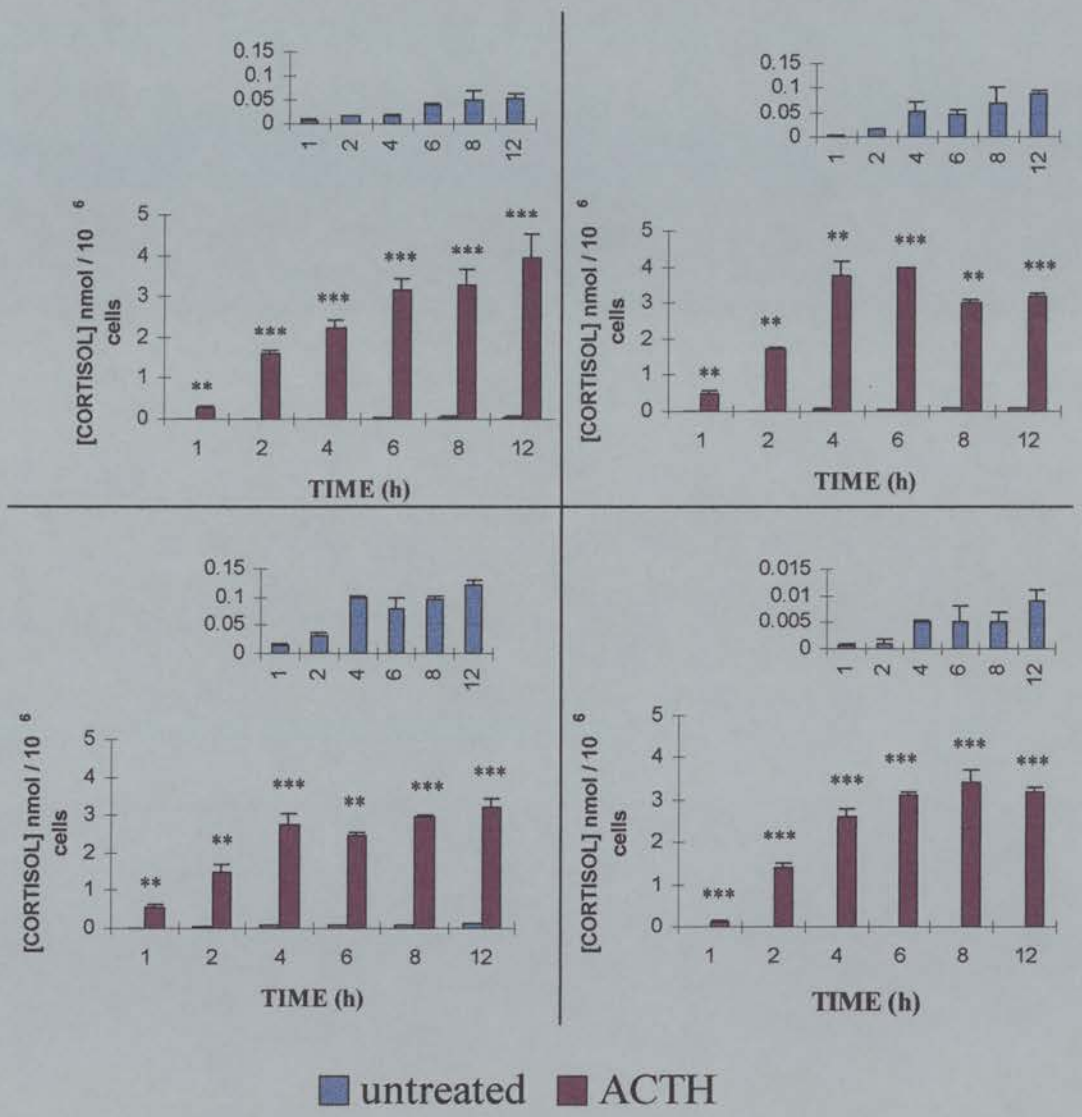


Figure 3.4A Cortisol production by BAC cells in response to $ACTH_{1-24}$ treatment. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) for the times indicated. The results presented are from four independent cell isolations, means ± SD from triplicate wells. **P<0.01, ***P<0.001 comparing untreated with ACTH-treated cells at each time point. Inserts show untreated cell data in more detail.

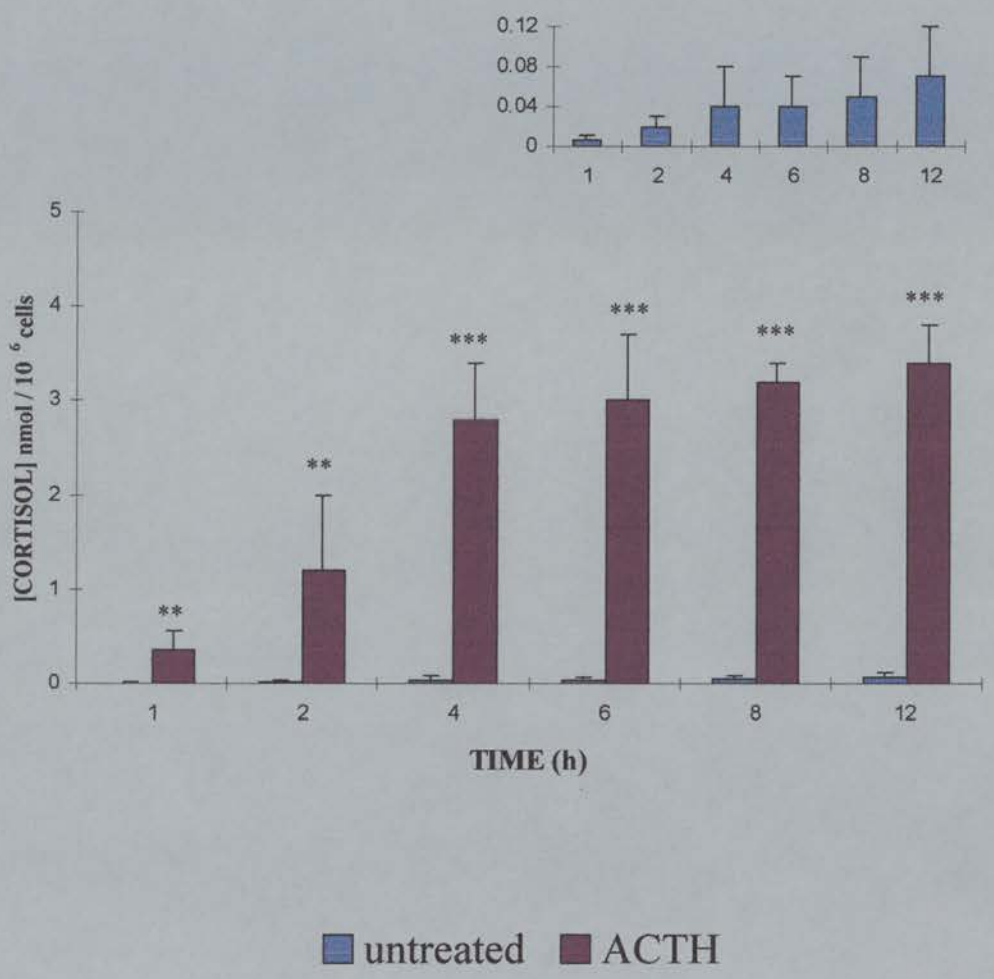


Figure 3.4 (B) : Cortisol production by BAC cells in response to $ACTH_{1-24}$ treatment. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) for the times indicated, medium overlying the cells was then removed and assayed for cortisol. Results shown are the combined mean ± SD of the four experiments in figure 3.4A. Insert shows the untreated cell data in more detail.
**P<0.01, ***P<0.001 comparing untreated with ACTH-treated cells at each time point.

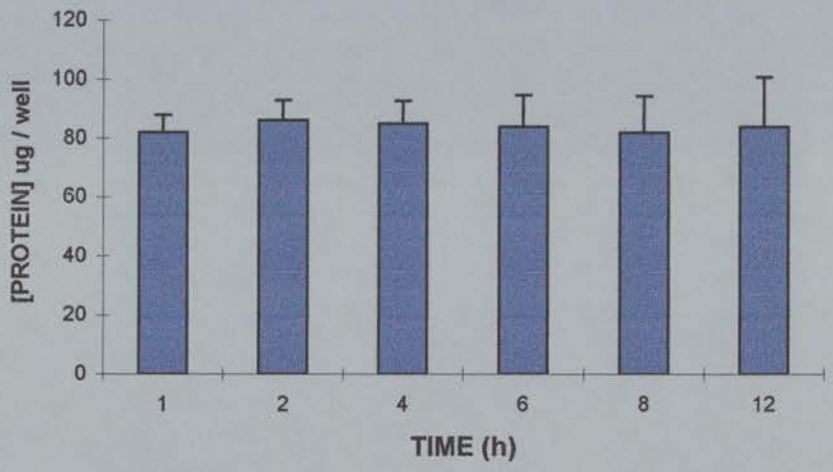


Figure 3.5 : Protein concentration of BAC cells incubated with $ACTH_{1-24}$. BAC cells were cultured for 2 days, serum-deprived overnight and incubated with $ACTH_{1-24}$ for the times indicated. The medium was removed and the cells solubilized in Triton X100 prior to assay for protein by the Bradford method (see section 2.2.5). Results shown are mean ± SD of three independent cell isolations.

3.2.4 Production of other steroids over the 12 h time course

HPLC data illustrated that over the 12 hour time period, $ACTH_{1-24}$-treated BAC cells produce four UV-absorbing, 4-en-3-ketosteroids: cortisol, corticosterone, cortisone and 11β-hydroxyandrostenedione. No steroids were detected in the untreated cells at any of the time points studied. Figure 3.6 shows representative traces from HPLC data at each of the time points.

Cortisol and corticosterone were detectable in the medium overlying BAC cells treated with 10nM $ACTH_{1-24}$ after 1 hour (figure 3.7). Over the first six hours of $ACTH_{1-24}$-treatment the amount of cortisol and corticosterone produced by BAC cells was similar, with no significant difference found. A significant difference was seen at 8 and 12 hours of $ACTH_{1-24}$ treatment as the amount of corticosterone produced by the cells decreased ($P<0.05$). Both cortisol and corticosterone output by BAC cells display the familiar pattern, i.e. an increase in cortisol production over the first 6 hours of ACTH treatment followed by a drop in output thereafter.

Cortisone and 11β-hydroxyandrostenedione were not detectable in the medium overlying BAC cells until 6 hours of $ACTH_{1-24}$ treatment. The cortisone output remained constant over the 6-12 hour period with no significant difference found at any time point, whereas 11β-hydroxyandrostenedione appears to peak at 8 hours; however, no significant difference was found. Cortisol production was found to be significantly increased over the production of cortisone after 6 ($P<0.01$), 8 ($P<0.05$) and 12 ($P<0.01$) hours $ACTH_{1-24}$-treatment. The levels of cortisol in the medium overlying $ACTH_{1-24}$-treated BAC cells were increased over those of 11β-hydroxyandrostenedione after 6 ($P<0.01$) and 12 ($P<0.05$) hours.

For the first 2 hours of ACTH-treatment, cortisol and corticosterone are produced in similar amounts (Table 4.1). Thereafter, the percentage of corticosterone found in

the medium decreases. The amount of cortisol secreted into the medium remains at around 50% of the total steroids detected, thus making it the principal steroid secreted by $ACTH_{1-24}$-treated BAC cells

The total amount of steroids in the medium overlying the BAC cells was also found to be increased over the initial 6 hours, followed by a decline in levels thereafter (figure 3.7E and table 3.1)

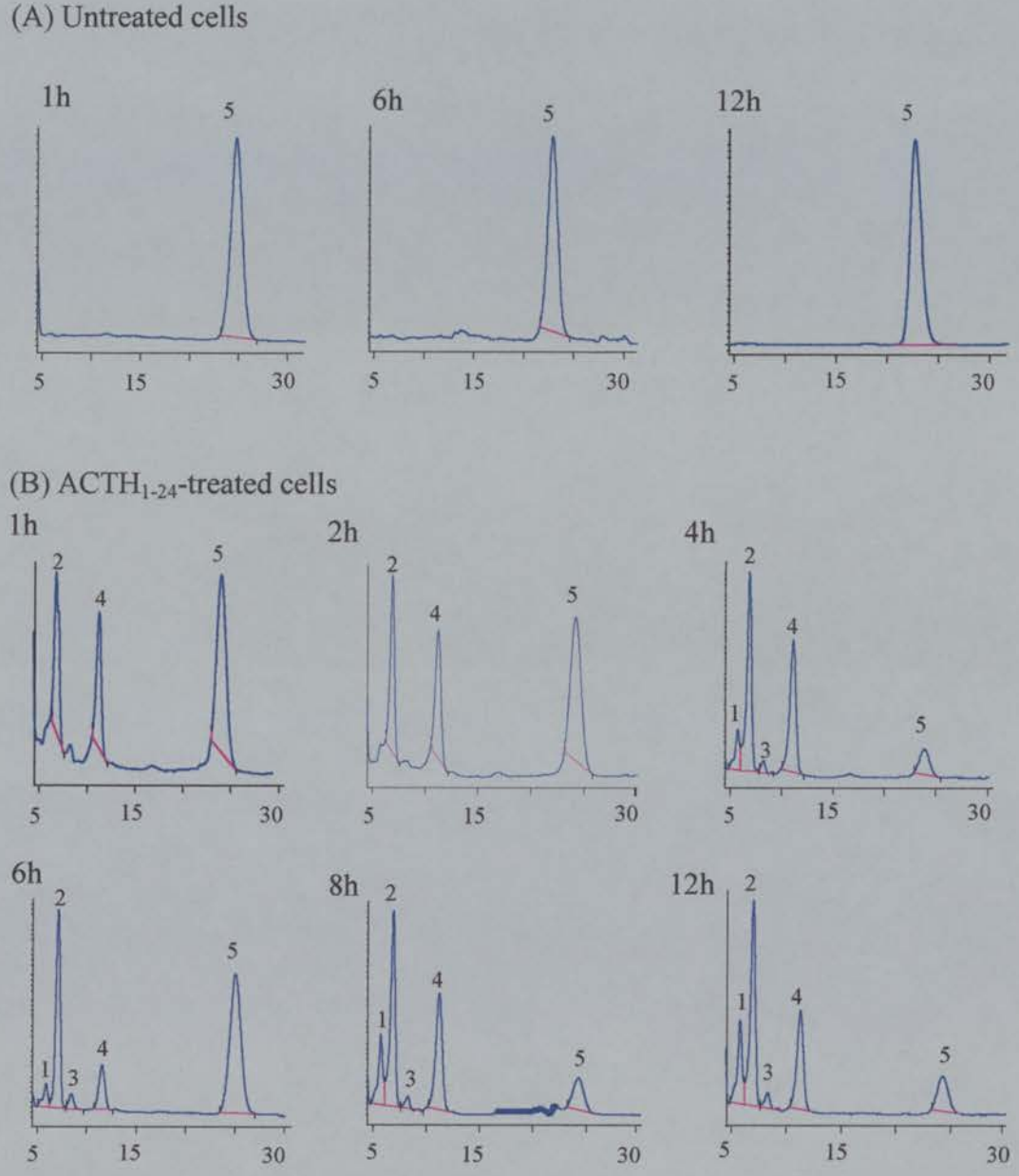


Figure 3.6 Representative HPLC traces of steroids secreted by BAC cells. Separation of adrenal steroids on C18μ Bondapak reverse phase column, absorptivity at 245nm. Peak 1 : cortisone, 2 : cortisol, 3 : 11β-hydroxyandrostenedione, 4 : corticosterone and 5 : testosterone (internal standard). The differences in peak height of the internal standard, testosterone, on the individual traces are due to the various amounts of testosterone added to the samples, ranging from 1 to 0.01nmole/ml, prior to extraction.

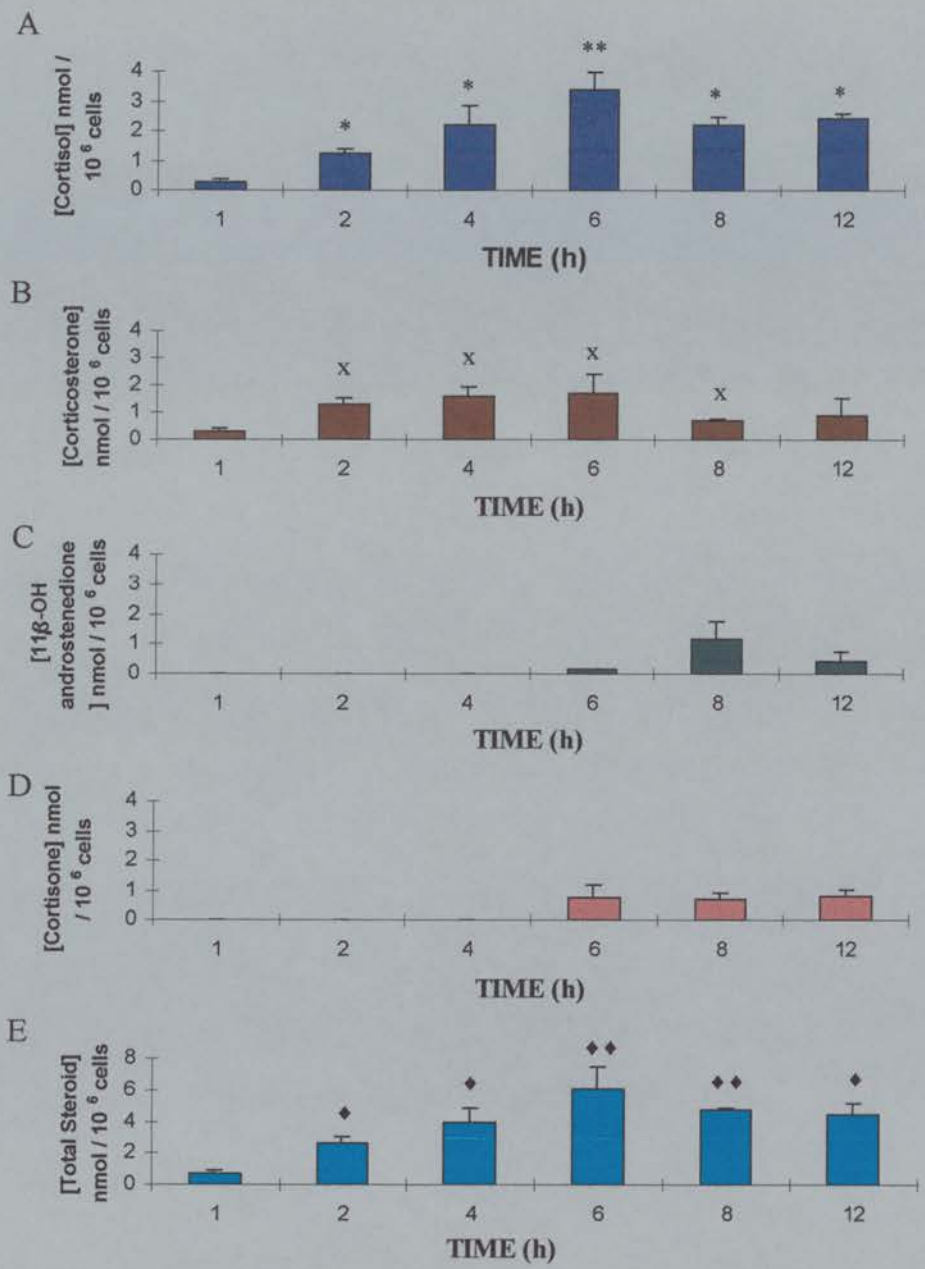


Figure 3.7 Distribution of UV-absorbing steroids produced by BAC cells in response to $ACTH_{1-24}$. Steroids produced by BAC cells, as measured by HPLC, are A, Cortisol; B, Corticosterone; C, 11β-hydroxyandrostenedione; D, Cortisone and E, Total steroid output. Results shown are means ± SD of three independent experiments. *P<0.05, **P<0.01, comparing all cortisol time points with the1 hour cortisol time point. x P<0.05 comparing all corticosterone time points with the 1 hour corticosterone time point. ♦ P<0.05, ♦♦ P<0.01 comparing total steroids at time points with 1hour time point.

STEROIDS (nmol/10^6 cells)	0h	1h	2h	4h	6h	8h	12h
Cortisone	0	0	0	0	0.8 (±0.4) (13%)	0.7 (±0.3) (14%)	0.8 (±0.3) (18%)
Cortisol	0	0.3 (±0.08) (50%)	1.3 (±0.2) (52%)	2.2 (±0.7) (58%)	3.4 (±0.6) (56%)	2.2 (±0.3) (46%)	2.4 (±0.2) (55%)
11β-hydroxy-androstenedione	0	0	0	0	0.2 (±0.03) (3%)	1.2 (±0.5) (25%)	0.4 (±0.3) (9%)
Corticosterone	0	0.3 (±0.1) (50%)	1.2 (±0.2) (48%)	1.6 (±0.3) (42%)	1.7 (±0.7) (28%)	0.7 (±0.1) (15%)	0.8 (±0.6) (18%)
TOTAL	0	0.6	2.5	3.8	6.1	4.8	4.4

Table 3.1 Distribution of steroids produced by BAC cells. Total amount of steroids produced by BAC cells (in parenthesis percentage of total steroid production). Results are mean ± SD from three independent cell isolations.

3.2.5 Pattern of cortisol production in response to various doses of $ACTH_{1-24}$

To investigate if the dose of $ACTH_{1-24}$ had any effect on the pattern of cortisol output by BAC cells, three further doses of $ACTH_{1-24}$ (0.01, 1 and 100nM) were used to treat the cells (figures 3.8-3.10).

Figure 3.8 displays the response of BAC cells to 0.01nM $ACTH_{1-24}$ treatment, where figure 3.8A displays three independent experiments and figure 3.8B the three experiments combined. The individual experiments, which were performed with triplicate wells for each time point, demonstrate that $ACTH_{1-24}$-treatment at all of the time points studied gave rise to a significant increase in the cortisol output in $ACTH_{1-24}$-treated cells compared with untreated cells. This was reflected in the combined results of figure 3.8B, where at each time point studied there was a significant increase in the cortisol secreted into the overlying of $ACTH_{1-24}$-treated cells compared with untreated cells ($P<0.05$).

Figure 3.9A & B displays the response by BAC cells to 1nM $ACTH_{1-24}$. For each of the three individual experiments (figure 3.9A) and the combined data (figure 3.9B), there was a significant increase in the amount of cortisol produced by $ACTH_{1-24}$-treated cells compared with untreated cells (see figures for details). Likewise, in response to 100nM $ACTH_{1-24}$ there was a significant increase in the cortisol content of the overlying medium compared with the untreated cells ($P<0.05$) (figure 3.10A & B)

The plateau in the cortisol output, when the experiments were combined, was found to be at 4 hours for 0.01nM, 6 hours for 1nM and between 8 and 12 hours for the highest dose, 100nM.

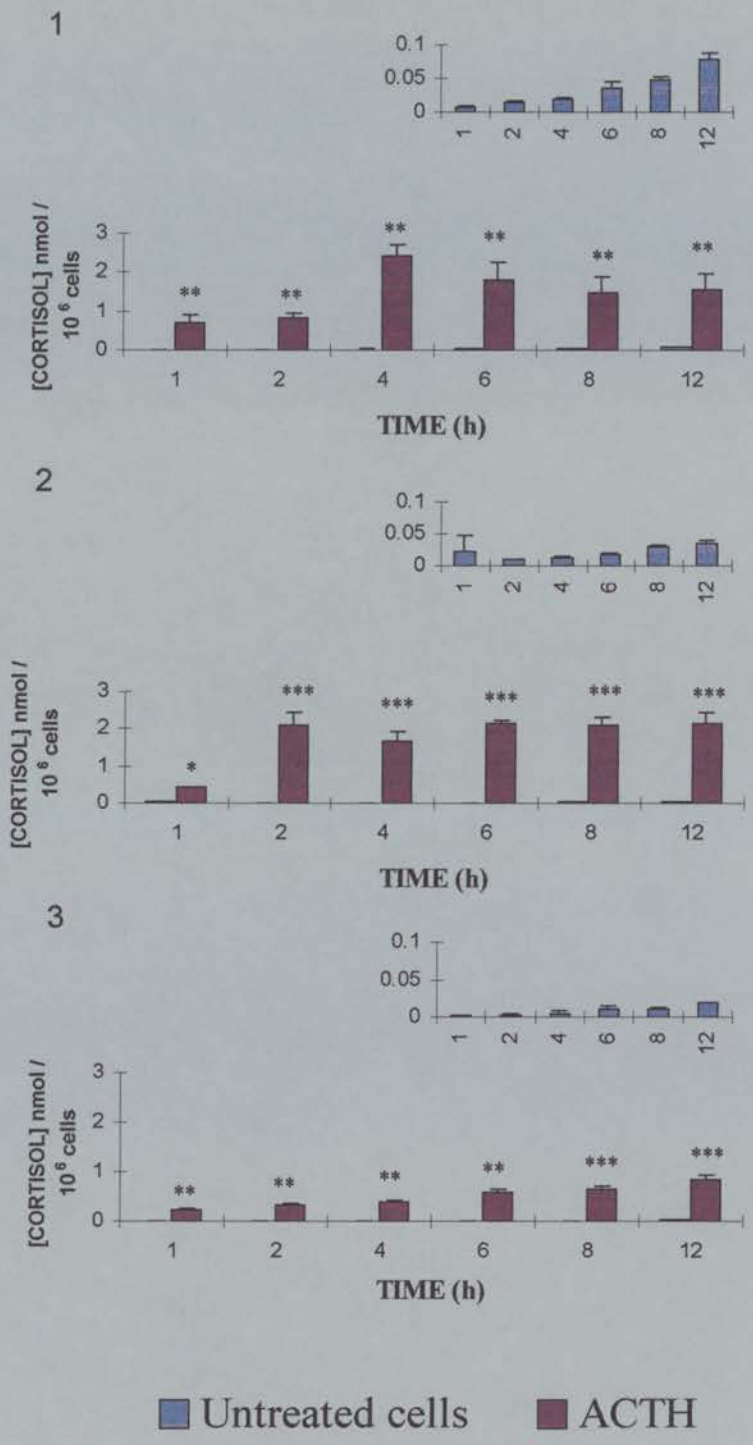


Figure 3.8A Cortisol production by BAC cells in response to 0.01nM $ACTH_{1-24}$-treatment. BAC cells were treated with or without $ACTH_{1-24}$ for times indicated. Results from three independent cell isolations (1, 2 &3) means ± SD from triplicate wells. *P<0.05, **P<0.01, ***P<0.001 comparing untreated with $ACTH_{1-24}$-treated cells at each time point. Inserts show untreated cell data in more detail.

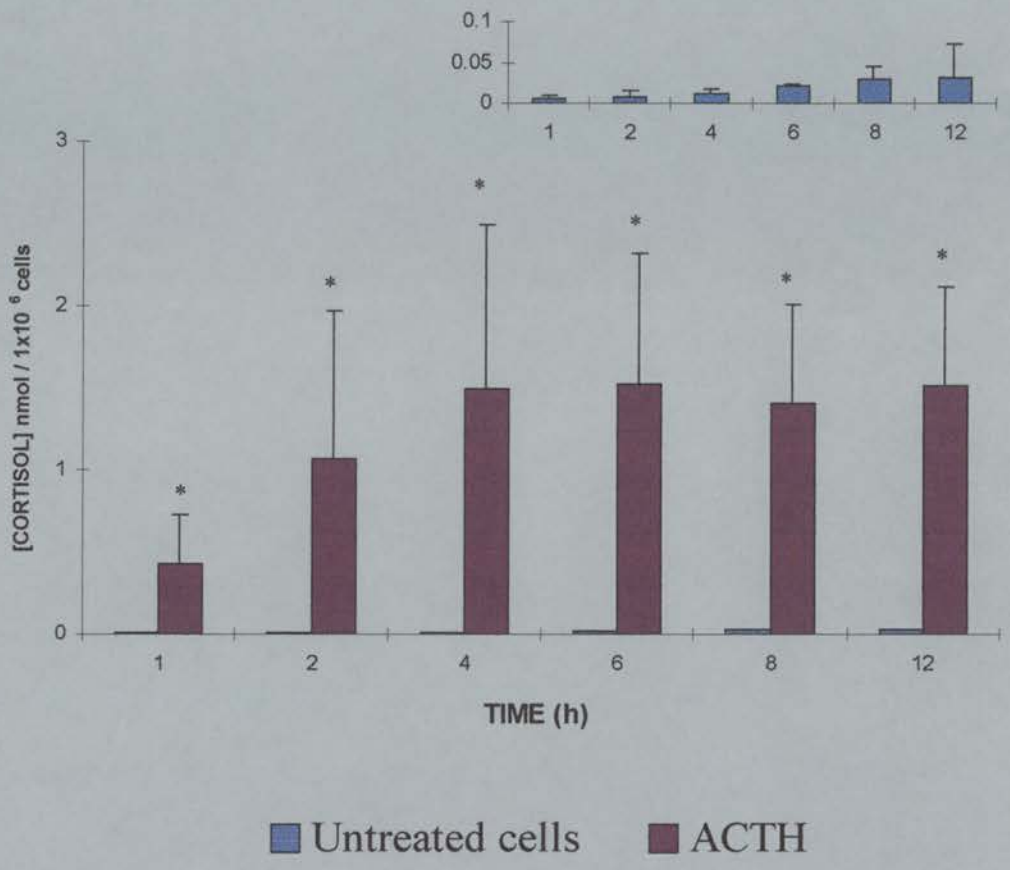


Figure 3.8 B : Response, by BAC cells, to 0.01nM $ACTH_{1-24}$
BAC were treated with $ACTH_{1-24}$ (0.01nM) for the times indicated. The graph on this page shows the combined mean ± SD of the three experiments shown in figure 3.8A. Inserts displays the untreated cells in more detail. *P<0.05 comparing untreated and $ACTH_{1-24}$-treated cells at each time point.

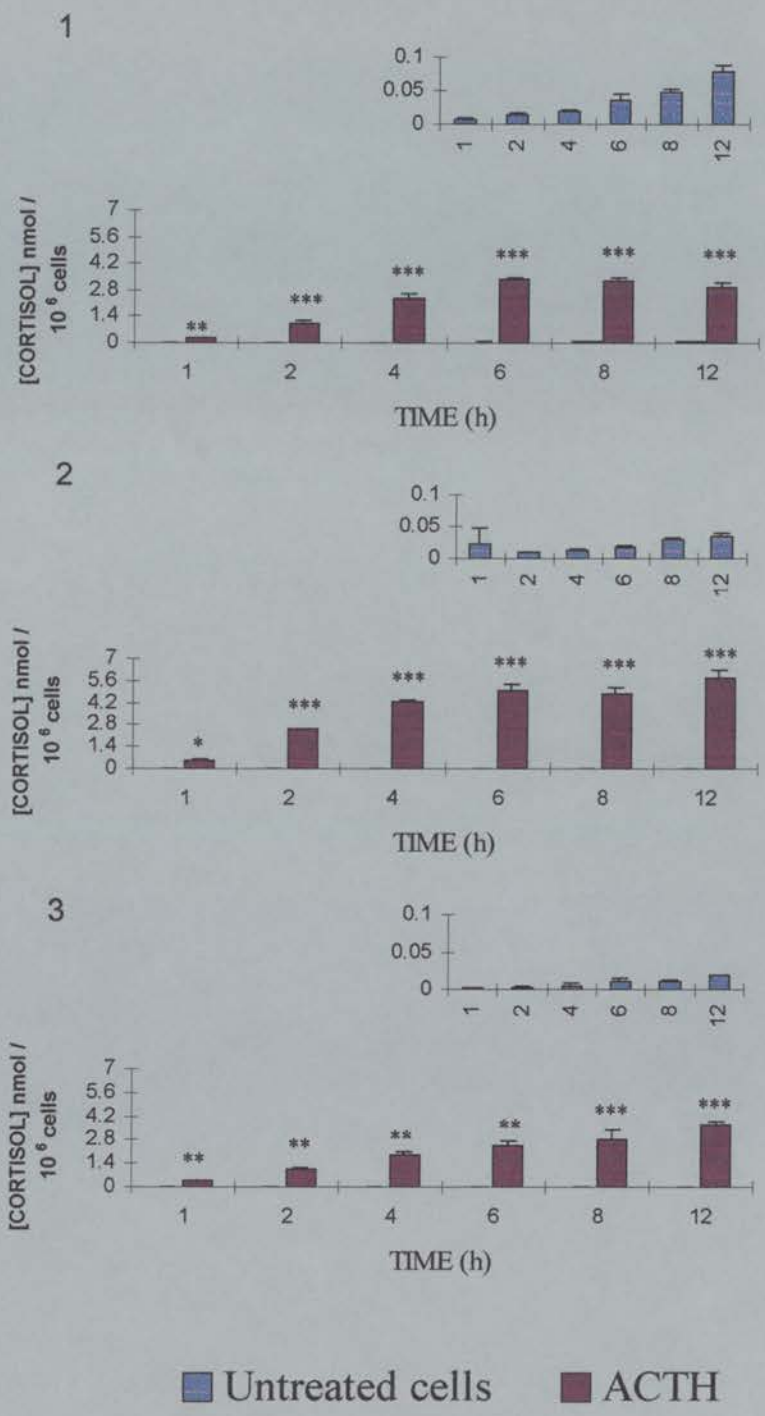


Figure 3.9A Cortisol production by BAC cells in response to 1nM $ACTH_{1-24}$-treatment. BAC cells were treated with $ACTH_{1-24}$ (1nM) for the times indicated Results from three independent cell isolations (1, 2 & 3), means ± SD from triplicate wells. *P<0.05, **P<0.01, ***P<0.001 comparing untreated with $ACTH_{1-24}$-treated cells at each time point. Insert shows untreated cell data in more detail.

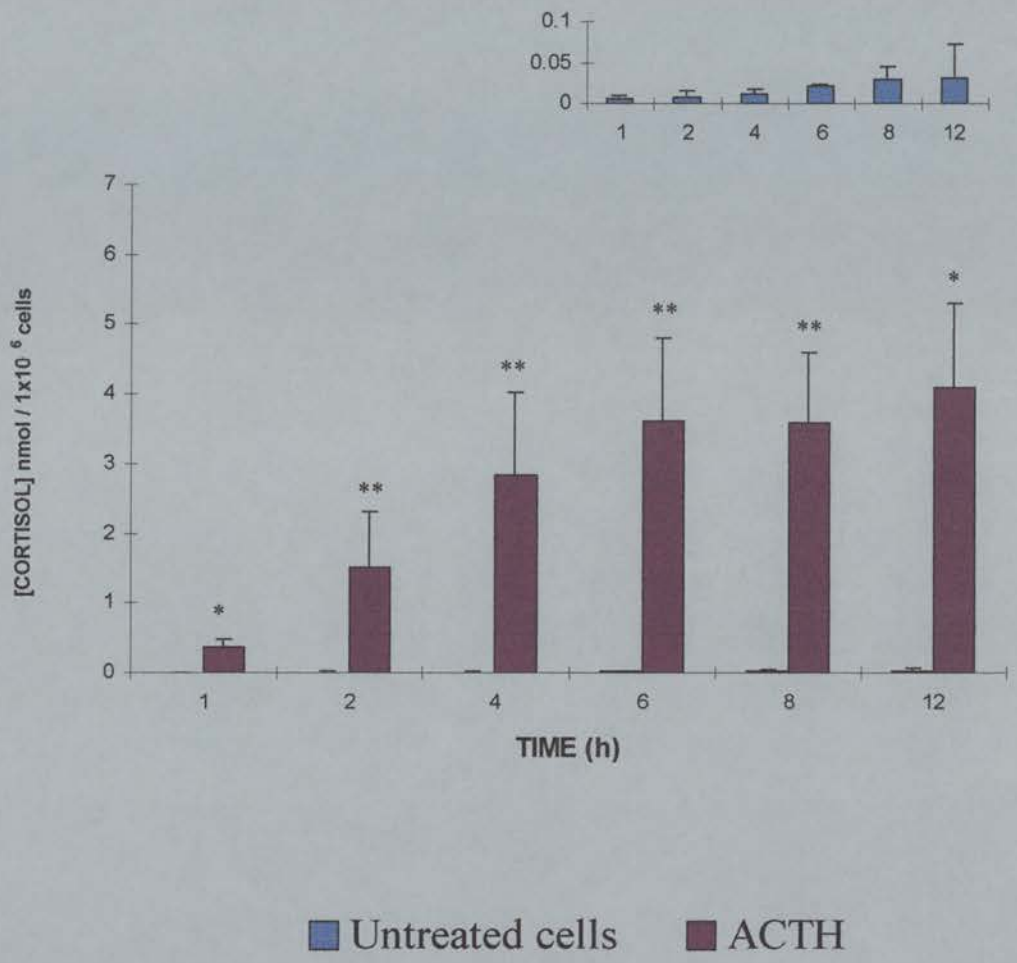


Figure 3.9B : Cortisol production by BAC cells in response to 1nM $ACTH_{1-24}$-treatment. BAC cells were treated with or without $ACTH_{1-24}$ (1nM) for the times indicated, the medium was removed and assayed for cortisol. Results shown are mean ± SD of the three independent experiments shown in figure 3.9A. Insert shows untreated cell data in more detail. *P<0.05, **P<0.01 comparing untreated with treated at each time point.

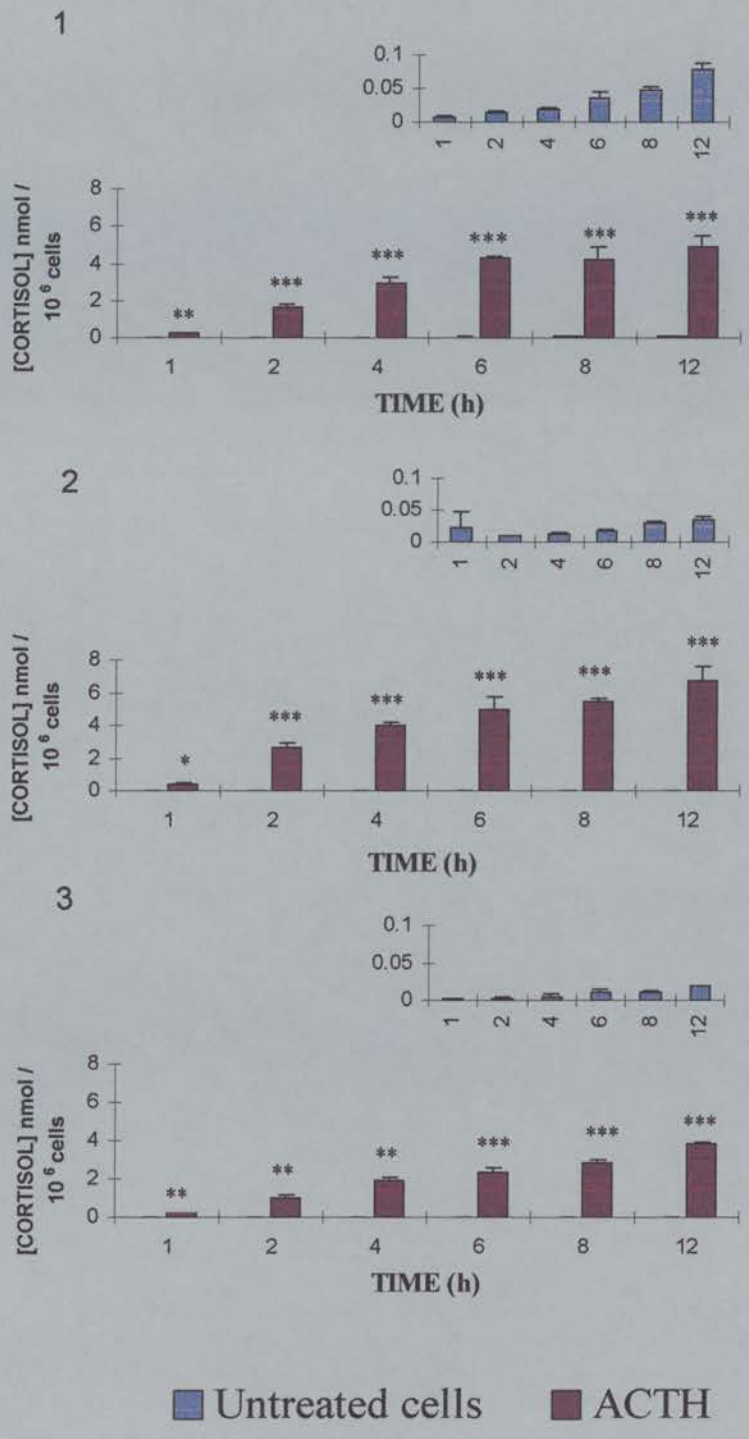


Figure 3.10A Cortisol output by BAC cells in response to 100nM $ACTH_{1-24}$-treatment. Results from three independent cell isolations (1,2 &3), means ± SD from triplicate wells. *P<0.05, **P<0.01, ***P<0.001 comparing untreated with $ACTH_{1-24}$-treated cells at each time point. Inserts show untreated cell data in more detail.

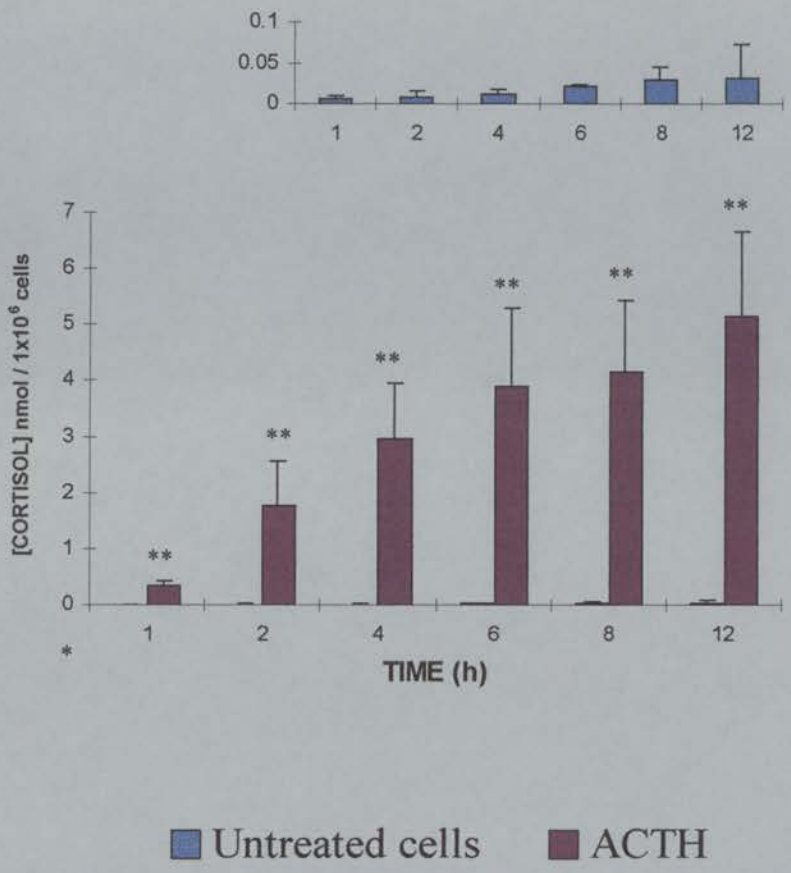


Figure 3.10B: Cortisol response, by BAC cells, to 100nM $ACTH_{1-24}$ treatment. BAC were cells were treated with or without $ACTH_{1-24}$ (100nM) for the times indicated, the medium was removed and assayed for cortisol. Results shown are mean ± SD of the three independent experiments shown in figure 3.10A. Insert shows untreated cell data in more detail. *P<0.05, **P<0.01 comparing untreated with treated at each time point.

3.2.6 Treatment of BAC cells with $ACTH_{1-39}$

The native ACTH peptide ($ACTH_{1-39}$) gave rise to a significant increase in the amount of cortisol secreted into the medium overlying BAC cells compared with untreated cells, at all of the time points studied ($P<0.001$) (figure 3.6). Treatment with $ACTH_{1-39}$ (10nM) produced a response comparable with that found for $ACTH_{1-24}$-treated (10nM) cells, for example at 6 hours of treatment values of 3.2 ± 0.6 and 3.3 ± 1.0nmol cortisol/10^6 cells were found with $ACTH_{1-24}$ and $ACTH_{1-39}$ respectively.

As with $ACTH_{1-24}$, the cortisol output by BAC cells in response to $ACTH_{1-39}$-treatment diminished over the time period studied. However the plateau began at a concentration of 3.8 ± 0.3nmol cortisol/10^6 cells, a level of cortisol found between 6 and 8 hours of treatment (3.3 ± 1.0 and 3.9 ± 1.1nmol cortisol/10^6 cells respectively).

These results establish that there was little difference in the response of BAC cells treated with $ACTH_{1-39}$ or $ACTH_{1-24}$. Consequently the use of the more economical and widely used synthetic $ACTH_{1-24}$ was valid, and this was used in subsequent experiments, unless stated otherwise.

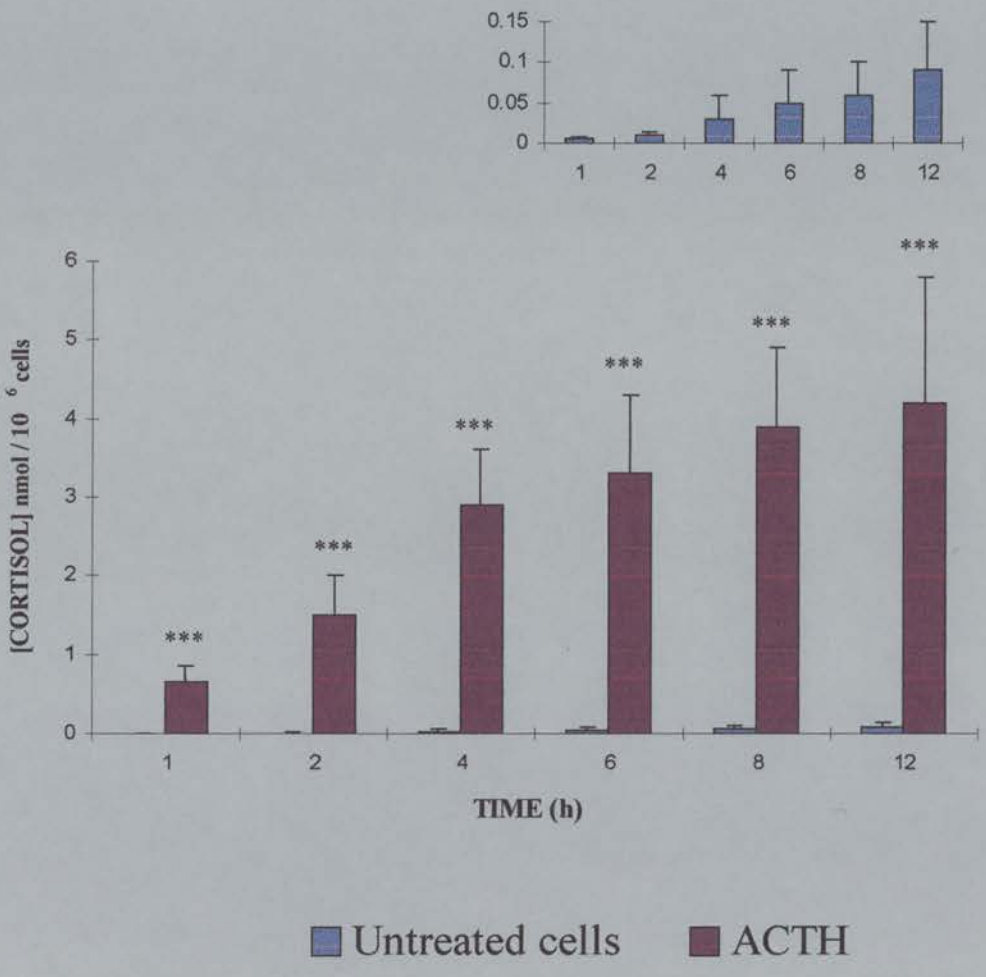


Figure 3.11 : Cortisol response in BAC cells treated with $ACTH_{1-39}$
BAC cells were treated with or without $ACTH_{1-39}$ (10nM) for the times indicated, the medium overlying the cells was then removed and assayed for cortisol. Results are mean ± SD of 5 independent cell isolations. Insert shows untreated levels in more detail. ***P<0.001 comparing untreated with $ACTH_{1-39}$-treated at each time point.

3.2.7 Response by BAC cells to Forskolin, 8 Br-cAMP and AngII

Forskolin-treatment gave rise to a significant increase in the amount of cortisol secreted into the medium overlying BAC cells, compared with untreated cells, at each of the time points studied; $P<0.01$ at 1 and 2 hours and $P<0.001$ at 4, 6, 8 and 12 hours (figure 4.3). When the data were analysed using a nonlinear curve fit equation no plateau in cortisol production was found. The maximal response in cortisol output was at 12 hours, when expressed as a percentage of this maximal response the other time points show a gradual increase in the amount of cortisol produced; 8% ± 5; 22% ± 7; 52% ± 11; 60% ± 5; 72% ± 13 at 1, 2, 4, 6 and 8 hours respectively.

Treatment of BAC cells with 8 Br-cAMP also produced a significant increase in cortisol secretion compared with untreated cells at each time point studied ($P<0.05$). Once more, no plateau in cortisol output was found when analysed by the nonlinear curve fit equation. As with forskolin the maximal response in cortisol output was at 12 hours and when expressed as a percentage of this maximal response the other time points show a gradual increase in the amount of cortisol produced; 4% ± 2; 15% ± 9; 47% ± 22; 60% ± 10 and 66% (± 15) at 1, 2, 4, 6 and 8 hours respectively.

AngII-treatment of BAC cells gave rise to a significant increase in cortisol output over untreated cells at all the time points studied ($P<0.05$). However, unlike forskolin and 8Br-cAMP, AngII-treatment of BAC cells produced a plateau in the cortisol output. Akin to that of $ACTH_{1-24}$, the plateau occurred around 6 hours of AngII treatment.

Figure 4.6 illustrates the different steroids produced by BAC cells in response to forskolin, 8 Br-cAMP and AngII. All four UV-absorbing steroids were found in response to forskolin-treatment. The profile of steroid output was similar to that

found with ACTH, in that cortisol and corticosterone are produced in similar amounts over the initial 4 hours, thereafter the cortisol levels increase and the levels of corticosterone fall. Cortisone was produced at 4 hours of treatment and appears to increase over the following 8 hours of treatment. The levels of 11β-hydroxyandrostenedione appeared to be less that those found with ACTH-treatment. Cortisol, corticosterone and cortisone were detected in the medium of 8Br-cAMP and AngII-treated cells. Levels of 11β-hydroxyandrostenedione were undetectable with both these treatments.

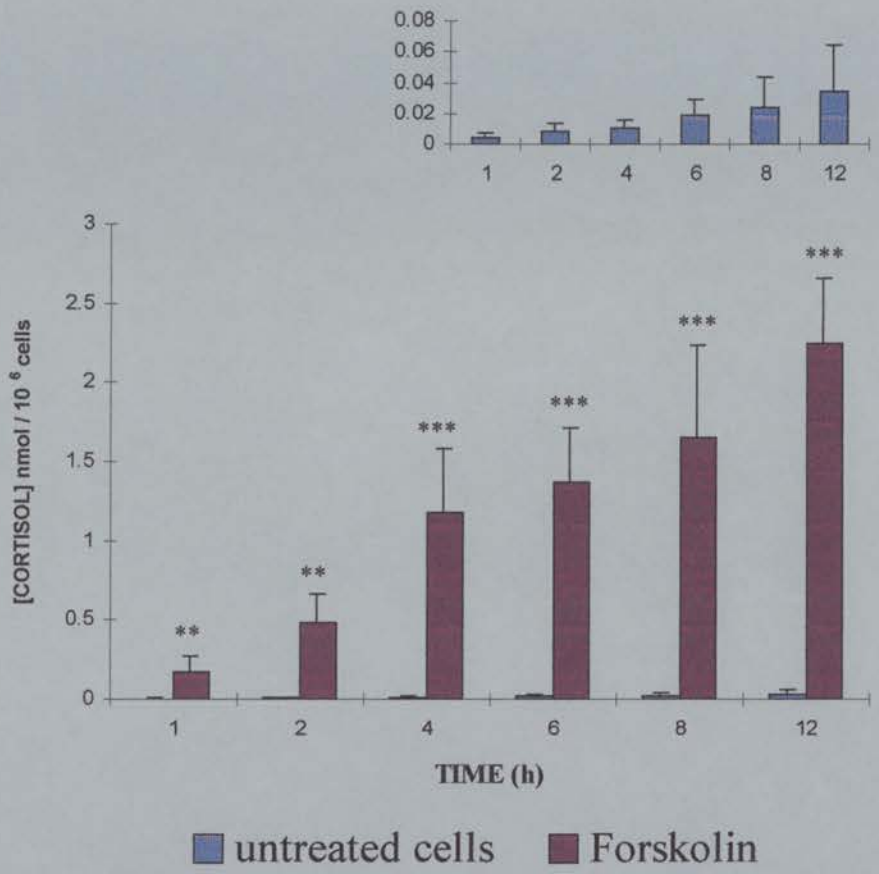


Figure 3.12 Cortisol output by BAC cells in response to forskolin (10 μM)
BAC cells were treated with or without forskolin for the times indicated, the experimental medium was removed and assayed for cortisol. Results shown are mean ± SD of five independent cell isolations. Insert shows untreated cell data in more detail.
**P<0.01, ***P<0.001 comparing untreated with treated at each time point.

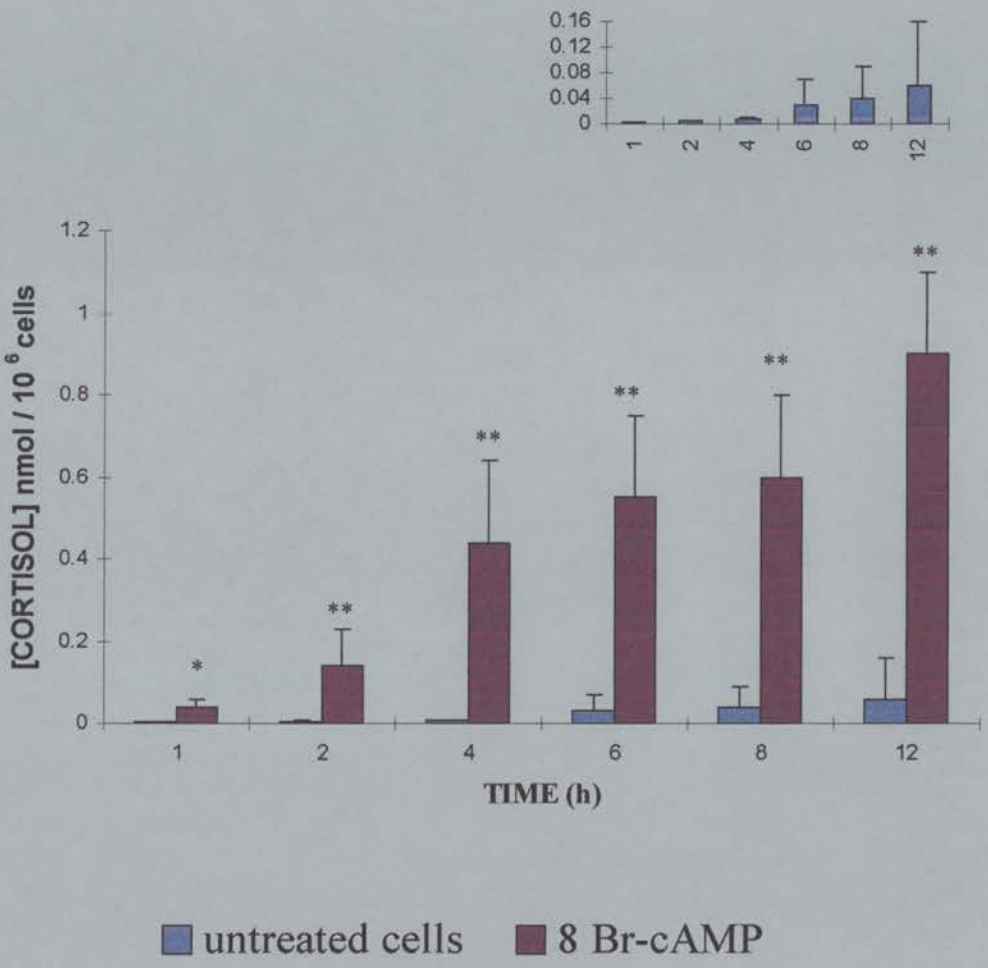


Figure 3.13 Cortisol output by BAC cells in response to 8-Br-cAMP (100 μM) BAC cells were stimulated with or without 8 Br-cAMP for the times indicated, the experimental medium was removed and assayed for cortisol. Results shown are mean ± SD of five independent cell isolations. Insert shows untreated cells in more detail. *P<0.05, **P<0.01, comparing untreated cells with treated cells at each time point.

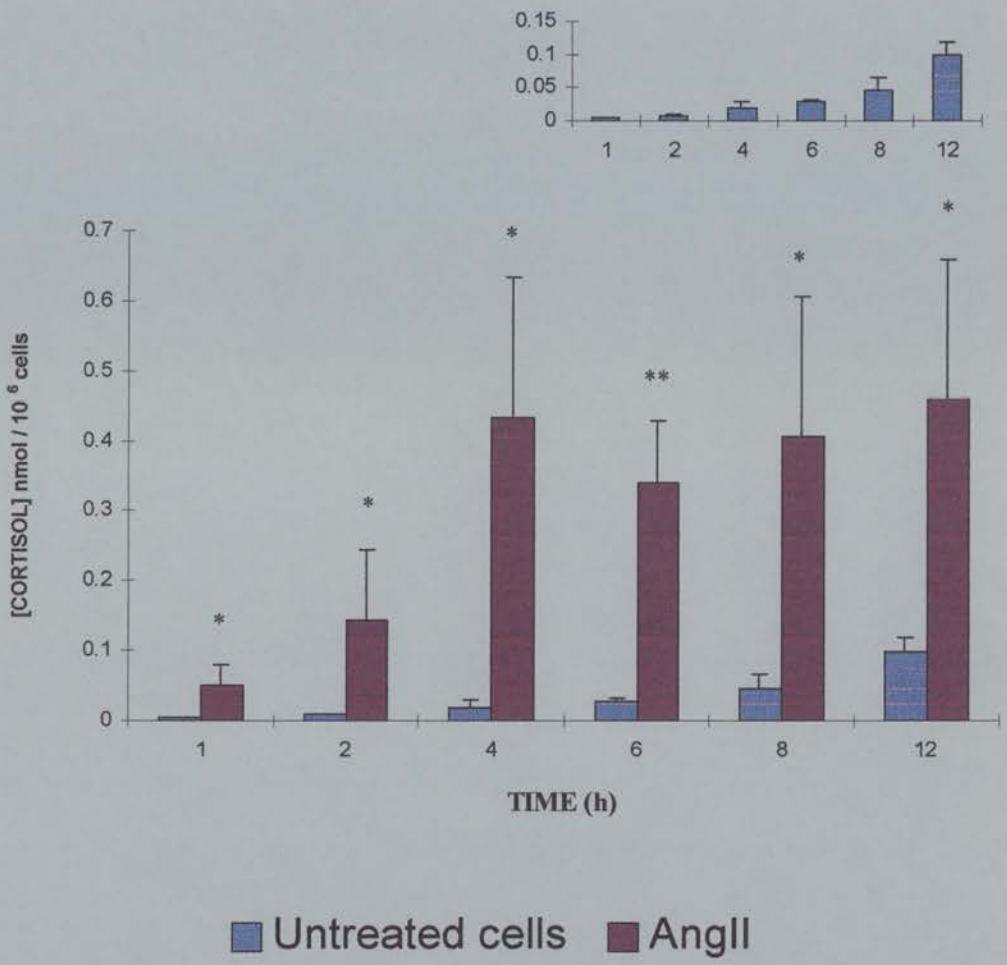


Figure 3.14 Cortisol output by BAC cells in response to AngII (10nM)
BAC cells were stimulated with or without AngII for the times indicated, the experimental medium was removed and assayed for cortisol. Results shown are mean ± SD of three independent cell isolations. Insert shows untreated cell data in more detail. *P<0.05, **P<0.01 comparing untreated cells with treated cells at each time point.

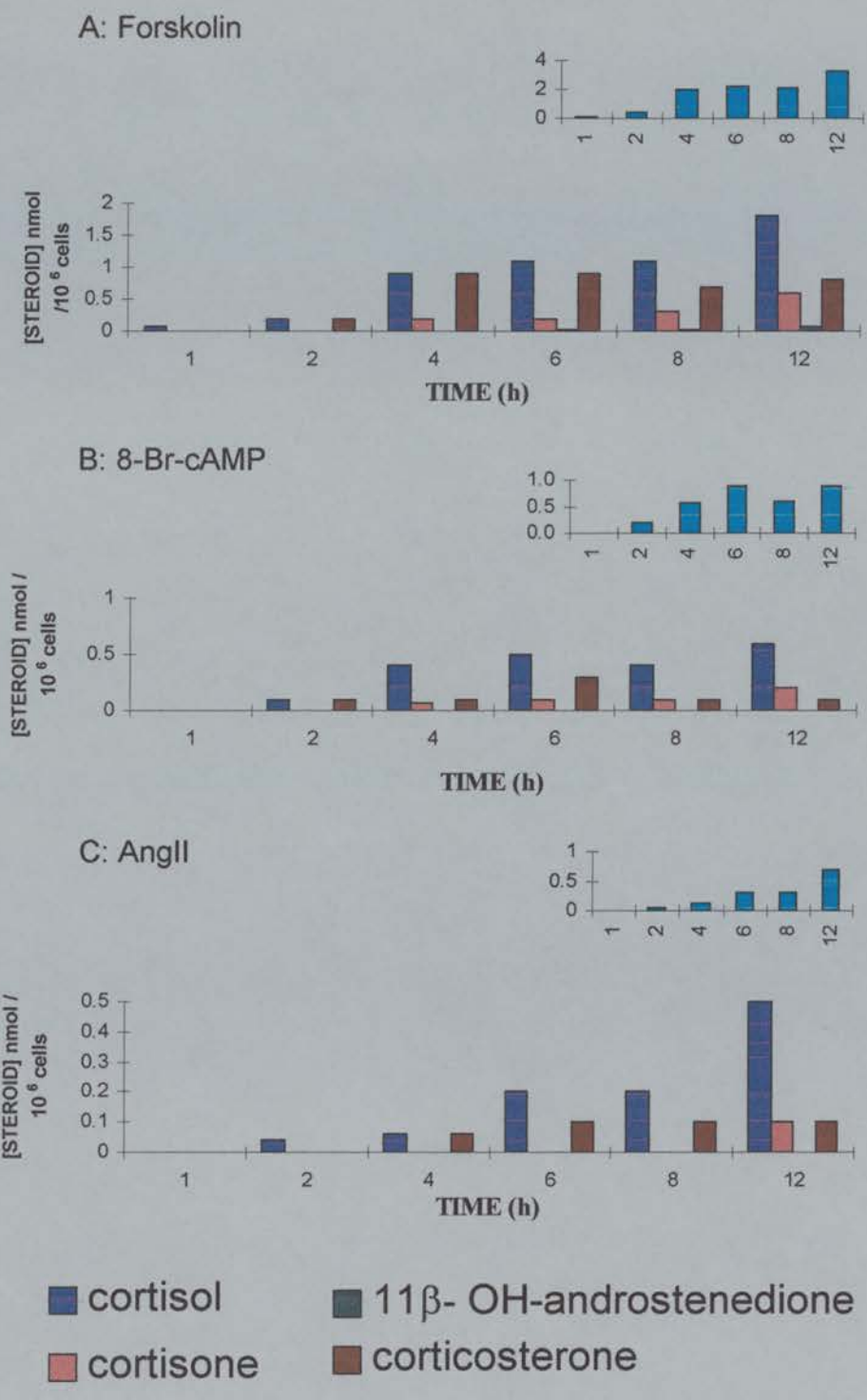


Figure 3.15 Distribution of UV-absorbing steroids produced by BAC cells. Steroids produced by BAC cells, as measured by HPLC, in response to A, Forskolin; B, 8 Br-cAMP and C, AngII. Total steroid output is shown in the insert graphs. Results shown are from one experiment.

3 3 Discussion :

Consistent with previous studies (Williams, 1989), (Bird, 1992a), the results presented here show an enhanced responsiveness of BAC cells to ACTH, when maintained in primary culture, compared to the response found with freshly isolated cells. Although exposure to $ACTH_{1-24}$ for 6 hours provokes a significant response from BAC cells on each of the days studied, the cells were less able to sustain such levels of output over 24 hour exposure to $ACTH_{1-24}$ on days 0 and 1. The cells improve with time in culture producing a significant cortisol response at 24 hour on days 2, 3 and 4 (figure 3.2). The increase in cortisol secretion in response to ACTH over the days of culture has been shown to be paralleled by increased formation of cAMP (Bird, 1992a). The response by BAC cells to other agonists such as angiotensin II, adrenaline and acetylcholine has also been shown to be enhanced with time in culture (Bird, 1992a) (Walker, 1991a).

Compared with freshly isolated cells, BAC cells were reported to show a marked increase in cellular lipid content following primary culture (Williams, 1989). Using electron microscopy it was found that after 48 hours in culture BAC cells showed improved integrity of ultrastructure with an increased density of lipid droplets. The number of lipid droplets on day 3 of culture was found to be six times the number of lipid droplets/per cell section of day 0 cells.

The increase in the number of lipid droplets within the BAC cell by day 3 of culture may be one of the reasons why the cells respond so well in lipid-free medium. The use of serum, whether as fetal calf serum or processed serum replacements, in cell culture is essential in the initial stages for effective plating and maintenance of the cells. However, to ensure that the response achieved was due to agonist treatment rather than any potential effect of hormones in serum, it is advantageous to use a more defined medium. Figure 3.3 demonstrated that the response by BAC cells to

ACTH in either serum-free or serum-containing medium was similar. An increase in the amount of cortisol secreted into the overlying medium was observed over the initial 6 to 12 hours thereafter, the further synthesis of cortisol was not detectable.

To investigate further this fall in the steroid response, BAC cells were treated with $ACTH_{1-24}$ over a time period of 12 hours, in lipid-free medium. Figure 3.4 shows that the diminished cortisol response occurs around 4-6 hours of $ACTH_{1-24}$-treatment. Similar results were found by Jefcoate et al, although in their study the cortisol output was found to be linear up to 12 hours after the addition of ACTH (100nM) (DiBartolomeis, 1984), (Jefcoate, 1987). The reason for the discrepancy could be the could be the presence of 10% fetal bovine serum in the medium and the higher concentration of ACTH used in these two studies by Jefcoate et al. The conclusion drawn by Jefcoate and colleagues for the decline in steroid output was a decrease in the amount of cholesterol availability to the mitochondria. On determining the cholesterol accumulation in BAC cell mitochondria, they found a peak accumulation (60%) was reached by 2 hour, followed by a dramatic decline in mitochondrial cholesterol, reaching untreated levels by 12 hours (DiBartolomeis, 1984). This was mirrored by an increase in cholesterol side-chain cleavage activity at 2 hours, followed by a decline in activity (Jefcoate, 1987). However, a study by Rainey et al showed BAC cells treated with ACTH for successive 12 hours could produce cortisol over a 72 hour period when maintained in lipoprotein-free medium. The cholesterol necessary as substrate was found to arise from *de novo* synthesis within the cell. ACTH was found to increase 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity within 12 hours of addition to the medium. (Rainey, 1985). The cholesterol content of the BAC cells used in this thesis was not measured. The lack of cholesterol availability is unlikely to be the reason for the decline in cortisol output as no decline in cortisol secretion was seen with forskolin or 8 Br-cAMP.

To exclude cell detachment as a cause of the decline in cortisol synthesis, the protein content of the wells was measured over the time-course of the experiment. The protein content was found to be constant over the 12 hour time course and cannot explain the plateau in the cortisol response (figure 3.5).

In an attempt to explain the plateau in cortisol production, the hypothesis that cortisol was either being converted to its inert metabolite, cortisone, or the accumulation of another steroid was investigated. Cortisol is converted to cortisone by the action of 11β-hydroxysteroid dehydrogenase (11β-HSD) in humans (Mazzocchi, 1998). 11β-HSD type 2 (11βHSD2), is an NAD-dependant unidirectional enzyme with high affinity for glucocorticoids (Funder, 1996). Recently the 11β-HSD2 cDNA was cloned in the bovine adrenal, showing a 67% homology with the human cDNA (Romero, 2000). HPLC was used to study UV-absorbing steroids secreted into the medium bathing the BAC cells. The data obtained reveal that over the 12 hour time period $ACTH_{1-24}$-treated BAC cells produce four principal steroids, cortisol, corticosterone, cortisone and 11β-hydroxyandrostenedione. Cortisol and corticosterone were produced in equal amounts for the first two hours after $ACTH_{1-24}$ addition, thereafter the levels of corticosterone decline and the levels of cortisol continue to increase up to 6 hours where a plateau of output is reached. The decline in corticosterone coincided with the detection of 11β-hydroxyandrostenedione in the medium overlying the BAC cells, which may reflect an increase in CYP17 activity. The CYP17 mRNA has been shown to increase in as little as 2 hours after ACTH treatment of BAC cells, with concentrations increasing 20-fold by 8 hours (Zuber, 1986). Cortisone was detectable in the medium after 6 hours of $ACTH_{1-24}$-treatment, around the time when a fall in the levels of cortisol output was observed. Nevertheless the levels of cortisone remaining constant over the following 6 hours. This suggests that some cortisol is converted to cortisone but not in sufficient quantities to explain the decline in the rate of cortisol synthesis. Other studies have also shown that BAC

cells produce cortisol, corticosterone and 11β-hydroxyandrostenedione, (DiBartolomeis, 1984), (Rainey, 1985). The results presented in this thesis and those of other researchers have suggested the following pathway for steroid production in BAC cells:

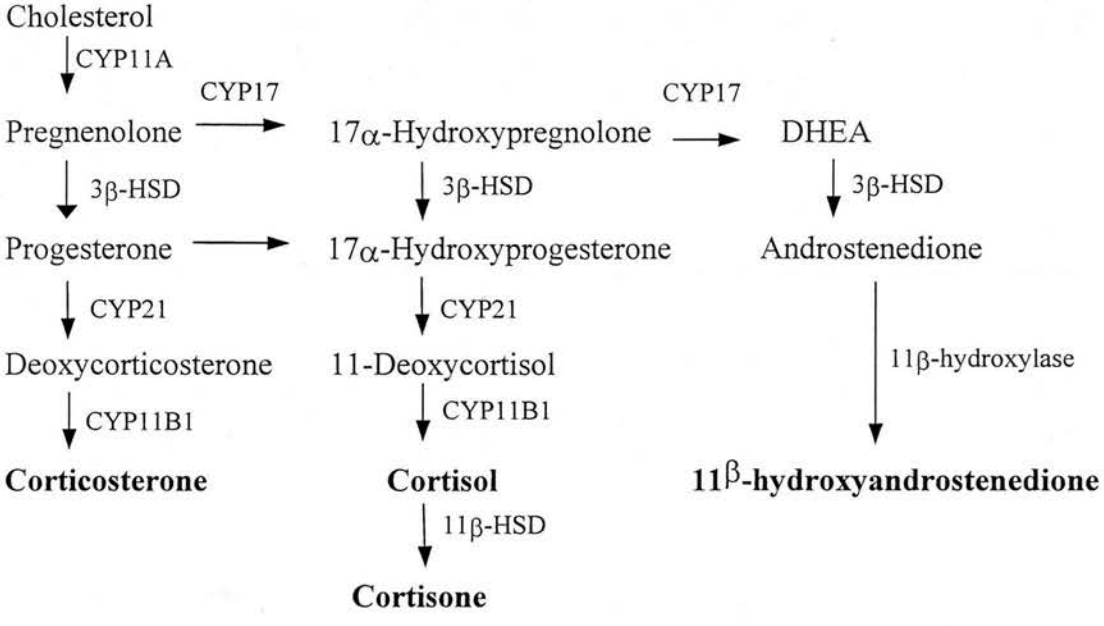


Figure 3.16 Pathway of steroid production in cultured BAC cells. The metabolism of cholesterol to the four main steroids (bold) in BAC cell culture.

The next step was to determine if the decrease in $ACTH_{1-24}$-treated BAC cells was dose-dependent. BAC cells were treated with three concentrations of $ACTH_{1-24}$, 0.01, 1, and 100nM. The output in cortisol with these doses of $ACTH_{1-24}$ was found to produce a similar pattern to that seen with 10nM $ACTH_{1-24}$. The plateau in cortisol production being reached earlier with the lowest concentration (0.01nM) of $ACTH_{1-24}$ and being less marked with the higher concentration (100nM). Thus suggesting that the ability of BAC cells to maintain cortisol output in response to $ACTH_{1-24}$ may be dose-dependent and that some factor is affecting the response at the lower concentrations.

$ACTH_{1-24}$ (Synacthen) is a synthetic hormone derivative containing full corticotropic activity used in place of ACTH for diagnosis and treatment. It is also used in the majority of steroidogenesis studies utilising cell culture as a model. To ascertain if the observed pattern in cortisol response was due to some artefact of $ACTH_{1-24}$, the full length peptide, $ACTH_{1-39}$, was used to treat BAC cells. $ACTH_{1-39}$ produced a comparable response to that found with $ACTH_{1-24}$, with an increase in the cortisol output up to 4-6 hours followed by a plateau in the production of cortisol thereafter. This indicates that $ACTH_{1-24}$ gives rise to a true representation of the steroidogenic response, thus the use of $ACTH_{1-24}$ was valid in these circumstances.

In order to determine if the plateau in cortisol output was restricted to ACTH action, the response of BAC cells to other agonists, forskolin, 8-Br-cAMP and AngII, was investigated. Both forskolin and 8 Br-cAMP utilise the cAMP second messenger pathway and AngII works via the phospholipase C pathway. If the reason for the plateau in cortisol production was related to the second messenger system then a corresponding decrease in the cortisol produced in forskolin- and 8 Br-cAMP-treated cells would be expected. Neither forskolin nor 8 Br-cAMP displayed a plateau in cortisol output over the 12 hour time period of the experiment. AngII on the other hand, showed a similar pattern to that found with ACTH, with a plateau in cortisol output being reached by around 6 hours. Thus, it appears that the plateau in cortisol production is not unique to ACTH-treatment and is not restricted to agonists acting via the cAMP second messenger pathway.

The results presented in this chapter have illustrated that the response of BAC cells to ACTH-treatment was maximal on day 3 of culture and this was in accordance with other studies (Williams, 1989). BAC cells respond to ACTH-treatment by secreting increasing amounts of cortisol into the medium bathing the cells over the initial 4-6 hours. Thereafter, the further synthesis of cortisol was not detectable. This decline in synthesis was not explained by the production of other steroids, as

cortisol remained the major steroids secreted over the 12 hour time course of the experiment. Treatment by forskolin and 8 Br-cAMP did not have the same effect on the production of cortisol, whereas AngII did. The action of forskolin and 8Br-cAMP are not mediated through receptors on the cell membranes. Therefore one possible explanation could be receptor desensitisation, as the actions of both ACTH and AngII are mediated via cell surface receptors (Raikhinstein, 1994), (Penhoat, 1995). As ACTH is the principal trophic hormone to stimulate the adrenocortical cells, the study of AngII was not taken further. Receptor desensitisation may be a result of growing BAC cells in primary culture. Primary culture is an artificial environment in which to grow cells and the responsiveness of the cells may be modified as a result. Further investigation of some aspects of the cell culture system would therefore be merited.

CHAPTER 4 : INVESTIGATION OF ACTH RECEPTOR DESENSITISATION AND SOME ASPECTS OF THE CELL CULTURE SYSTEM WHICH MAY EFFECT THE STEROIDOGENIC RESPONSE BY BAC CELLS

4.1 Introduction

The ACTH receptor was one of the earliest receptors to be recognised when Haynes (1958) described the action of ACTH in generating cAMP in adrenal cells (Haynes, 1958). The cloning of the human ACTH receptor has allowed the definition of a new subfamily of G protein-coupled receptors, the melanocortin receptor family, of which there are five members, MC1-5R (Mountjoy, 1992). The MC2-receptor (MC2-R) preferentially binds ACTH and is expressed in the adrenal cortex. Thus it is considered to be the ACTH receptor (Liakos, 1998). The deduced bovine ACTH receptor sequence shows 81% homology to the human receptor (Raikhinstein, 1994). Both the human and bovine sequences comprise 297 residues (MW 33258kDa) and show seven hydrophobic transmembrane domains. Amino acids within the membrane spanning domains are important for ligand recognition. ACTH binds through amino acid residues 1-18, thereby initiating the chain of events that mediate its function. Analysis of the bovine and human ACTH receptors revealed both protein kinase A (PKA) and protein kinase C (PKC) phosphorylation motifs, suggesting that the involvement of PKA and PKC pathways in ACTH action may be mediated in part by phosphorylation of the ACTH receptor (Raikhinstein, 1994).

The technique of cell culture allows cells to be maintained outside the body and given the appropriate conditions cells will not only survive but multiply and even express differentiated properties. However, cell culture systems do not provide the complete three-dimensional structure, cell-cell interactions, blood supply,

extracellular matrix and paracrine influences that the cells have *in vivo*. Primary cell cultures comprise cells derived directly from a particular organ or tissue and as such are generally considered to be more representative than cell lines in expressing tissue-specific properties. However, the loss of morphological and biochemical characteristics associated with the tissue of origin can occur during the process of adaptation to tissue culture conditions. The extent of this dedifferentiation depends on the cell type and its suitability to the culture conditions. An important factor is the nutritional environment which will affect all physiological and metabolic events occurring within the cells. Culture media contains various components present in body fluids e.g. the correct balance of salts, vitamins, amino acids necessary for cell functions and an energy source in the form of six carbon sugars, such as glucose. for the growth of cells in primary culture. The requirement for an animal serum (fetal calf, horse or a serum replacement mix) is necessary for growth factors, essential hormones and attachment factors.

Primary cultures of adrenocortical cells have been invaluable in many studies of the molecular biology, cell biology and physiology of steroidogenesis within the adrenal cortex. In BAC cell culture care must be taken in order to minimise the degree of contamination of cells from the zona glomerulosa and medulla which may affect the outcome. Contamination with non-steroidogenic cells such as fibroblasts does not usually occur in BAC cell culture. One advantage of using bovine adrenal glands over human glands is the clear zonation found in the bovine, allowing the isolation of the cortical cells at high purity. Careful dissection of the adrenal zones and purification of the dispersed cells by density gradient centrifugation or column filtration to remove debris (see 2.2.1) helps to minimise contamination (Hornsby, 1991).

A study by Hornsby et al illustrated that BAC cells could be maintained in culture with a life span of about 60 generations (Hornsby, 1978). The morphology of the

cells did not change greatly and the cells retained the ability to synthesise steroids in response to ACTH-treatment over the time culture. BAC cells in continuous primary culture have been shown to synthesise cortisol initially as the major steroid. After 10 to 20 days of culture, cortisol synthesis was no longer detected and could not be stimulated by ACTH (Simonian, 1979). The levels of deoxycortisol and earlier precursors increased, thus providing evidence that BAC cells lose the ability to 11β-hydroxylate steroids with time in culture.

The results presented in chapter 3 suggested that the plateau in cortisol production found in ACTH-treated BAC cells may be the result of ACTH receptor desensitisation. A series of experiments were undertaken in this chapter to investigate this possibility. Also investigated in this chapter was the stability of ACTH itself. ACTH has a short plasma half-life (7-12 minutes) (Aron, 2000a), although the synthetic ACTH (synacthen) is reported to be more stable.

Also investigated in this chapter was the effect of the cell culture medium on the ability of the BAC cells to maintain a steroidogenic output.

4.2 Results

4.2.1 Repeat $ACTH_{1-24}$ treatment of BAC cells.

An experiment was designed to verify that the cells were still responsive after 24 hours $ACTH_{1-24}$-treatment. One set of BAC cells were incubated for 24 hours with $ACTH_{1-24}$ (10nM), the other set were treated as normal, i.e. no $ACTH_{1-24}$ pre-incubation. The following morning both sets of cells were washed twice with EBSS and treated with $ACTH_{1-24}$ (10nM) for various times between 1 and 12 hours.

Figure 4.1 demonstrates that 24 hour of $ACTH_{1-24}$-treatment had no effect on the responsiveness of the cells to further ACTH treatment. The red bars represent the primary response to $ACTH_{1-24}$ (10nM) treatment over the time period studied i.e. cells which have had no ACTH pre-incubation. The green bars shows the secondary response i.e. cells incubated overnight with $ACTH_{1-24}$ (10nM) and then subjected to $ACTH_{1-24}$ for the same times as the primary experiment. A significant increase in the cortisol response over untreated cells was seen under both conditions ($P<0.05$).

The amount of cortisol secreted into the overlying medium was slightly higher in the cells pre-treated with $ACTH_{1-24}$ for 24 hours, though this was not found to be significant at the early time points. However, BAC cells pre-incubated with $ACTH_{1-24}$ for 24 hours displayed a significant increase in the amount of cortisol produced at both 8 and 12 hours of $ACTH_{1-24}$-treatment compared with the cells that were not pre-incubated with $ACTH_{1-24}$ ($P<0.05$).

The timing of the cortisol plateau was slightly later in the $ACTH_{1-24}$ pre-incubated cells. For the cells which where not pre-incubated with ACTH the plateau occured at a cortisol concentration of 1.5 ± 0.1nmol/10^6 cells, somewhere between 4 and 6 hrs $ACTH_{1-24}$ treatment. In contrast, the cells pre-incubated with $ACTH_{1-24}$ showed a

plateau forming at 2.2 ± 0.1nmol/10^6 cells, which is between 6 and 8 hours of $ACTH_{1-24}$-treatment.

4.2.2 Effect of 12 hour $ACTH_{1-24}$ treatments on BAC cells.

BAC cells were grown in 6-well plates as previously described and serum-deprived overnight. The cells were washed twice with EBSS and the medium with or without $ACTH_{1-24}$ replaced. The medium was then changed every 12 hours, through 72 hours. The medium sample at each time point was stored for cortisol measurement, and the cells removed from the culture plate with Triton X-100 for protein determination (see methods, 2.2.5).

As can been seen from figure 4.2, BAC cells continued to respond to $ACTH_{1-24}$ over the 72 hour period of treatment. After each 12 hour replenishment there was a significant amount of cortisol generated by the BAC cells treated with $ACTH_{1-24}$ compared with untreated cells, at each time point $P<0.001$. There was a significant decrease in the amount of cortisol produced between the 12 and 24 hour period, 3.9 ± 0.2 and 2.4 ± 0.3nmol/1x10^6 cells at 12 and 24 hours respectively ($P<0.01$). Over the remaining 72 hours the cortisol output increased again to roughly the 12 hour value, 3.4 ± 0.6nmol/1x10^6 cells at 72 hours. This value was not significantly different from that at 12 hours.

To verify that the cell number remained constant throughout the term of the experiment the protein content of the tissue culture wells were measured at each time point. Figure 4.2B confirms that there was no significant difference in the protein content at any given time during the course of the experiment.

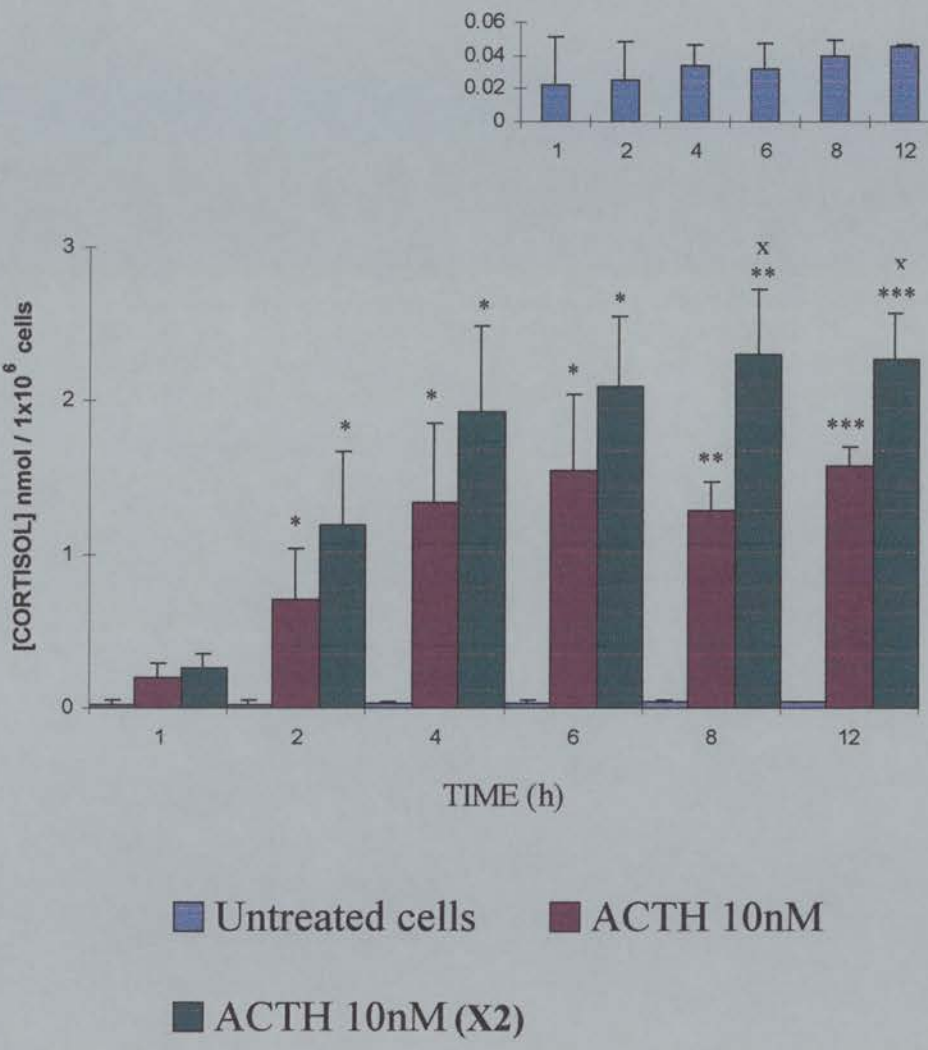


Figure 4.1 Treatment and re-treatment with $ACTH_{1-24}$ BAC cells were either treated without $ACTH_{1-24}$ (maroon bars) or with $ACTH_{1-24}$ (green bars) overnight prior to treatment with $ACTH_{1-24}$ for times indicated. Results are mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 comparing untreated with treated for each condition at each time point. ^{x}P<0.05 comparing $ACTH_{1-24}$ conditions at each time point studied. Insert shows untreated cell data in more detail.

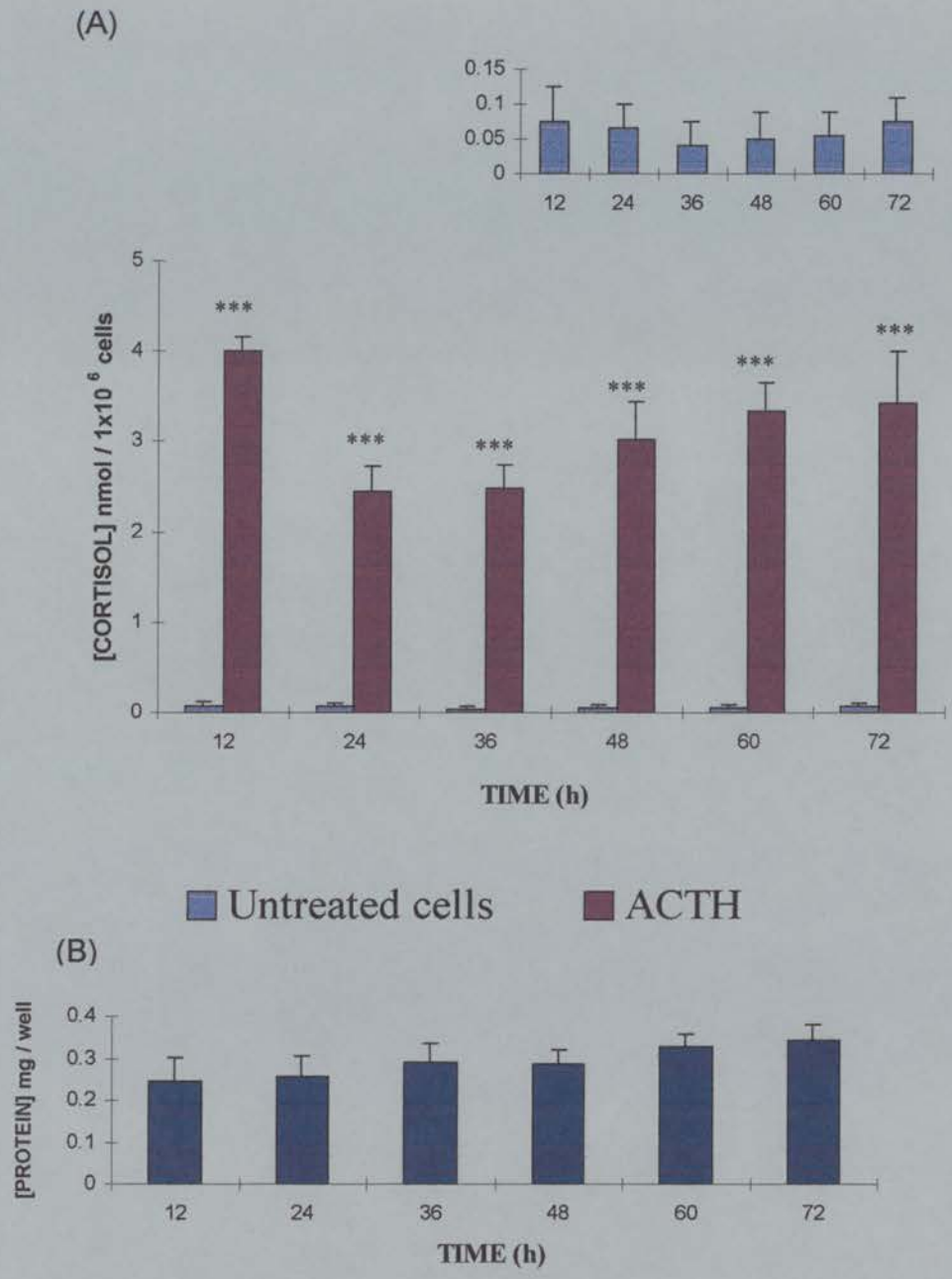


Figure 4.2 BAC cells treated with $ACTH_{1-24}$ every 12 hours. BAC cells are cultured for 2 days and serum-deprived overnight. The medium, with or without $ACTH_{1-24}$, overlying the cells was replenished every 12 hours. Results shown in graph A are mean ± SD of four independent cell isolations. *** $P<0.001$, comparing untreated with treated conditions. Insert displays the untreated cells in more detail. Graph B displays the attached cell protein concentration, of $ACTH_{1-24}$-treated cells, throughout the experiment.

4.2.3 Medium Glucose concentration.

To ascertain whether Ham's F10 has an adequate glucose concentration (1g/l) to cope with BAC cell steroidogenesis over the time period studied, DMEM/F12, a medium containing a higher glucose concentration (3g/l) was used. One set of BAC cells were maintained in DMEM/F12 containing 10% CPSR1 for 2 days and serum-deprived over-night in DMEM/F12 + 0.2% BSA, while the other set was maintained in Ham's F10 as previously described. The following morning the cells were washed twice with EBSS and then treated with $ACTH_{1-24}$ (10nM) in the appropriate medium, with 0.2% BSA added, for various times between 1 and 12 hours.

In response to $ACTH_{1-24}$-treatment BAC cells grown in DMEM/F12 produced a comparable response to that found in cells grown in Ham's F10 medium. The $ACTH_{1-24}$-treated cells produced a significant response when compared to the untreated cells at each of the time points studied, $P<0.05$ at 1hour and $P<0.01$ for the remaining time points (figure 4.6). No significant difference was seen between the two conditions at any of the time points. The untreated cortisol values for cells maintained in DMEM/F12 are shown as an insert, the cortisol output by untreated cells in both mediums was indistinguishable.

Cells grown in DMEM/F12 thus display the same pattern as cells grown in Ham's F10 medium in that the cortisol output increases over the initial 4 hours, reaching a plateau around 6 hours of $ACTH_{1-24}$-treatment. For cells maintained in both Ham's F10 and DMEM/F12 the plateau in cortisol output was found to occur at the same time, around 8 hours.

The glucose content in Ham's F10 medium bathing BAC cells was monitored throughout the time course. At 0 hours the glucose concentration in the Ham's F10 was 5.8mmol/l. The glucose concentrations at 6 hours in $ACTH_{1-24}$-treated and untreated cell medium was 5.6mmol/l (97% of amount at 0h), and again at 12 hours

the glucose concentrations in the medium overlying both untreated and treated cells was 5.55mmol/l (96 %) and 5.4mmol/l (93 %) respectively. This demonstrates that the amount of glucose taken up into the cells as an energy source for steroidogenesis was a small proportion of that present in the medium overlying the cells.

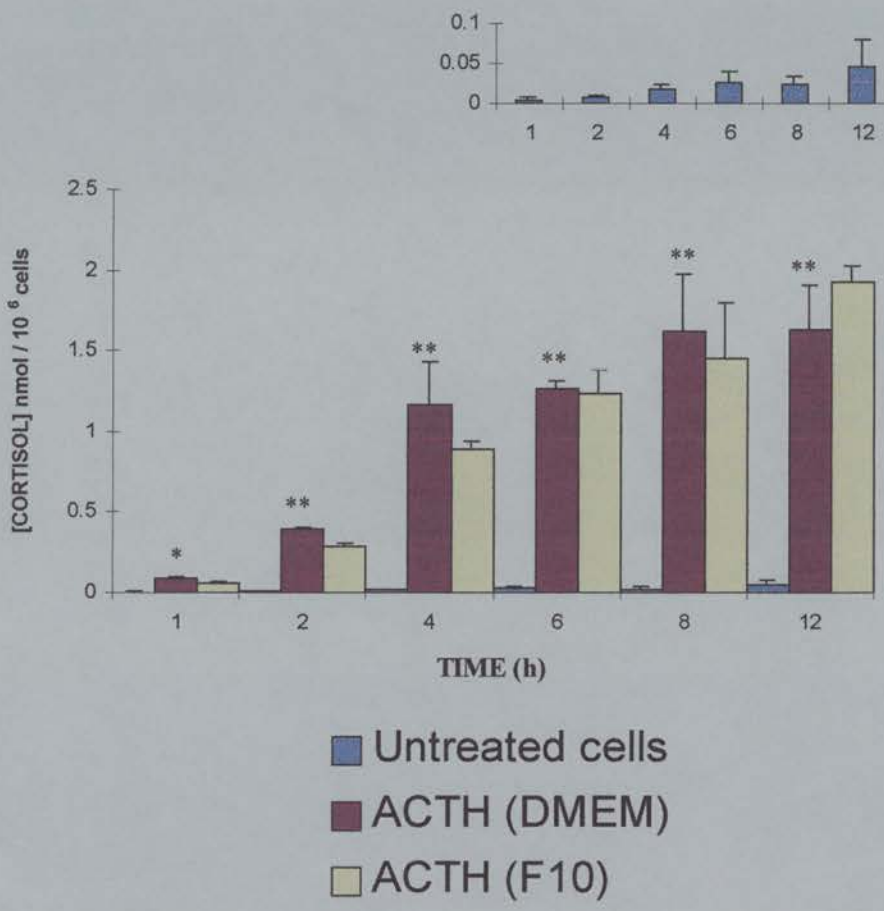


Figure 4.3 $ACTH_{1-24}$ (10nM) treatment of BAC cells in DMEM/F12. BAC cells were either maintained in DMEM/F12 (maroon bars) or Ham's F10 (yellow bars) and treated with or without $ACTH_{1-24}$ 10nM for the time indicated. The medium overlying the cells was removed and assayed for cortisol content. Results shown are mean ± SD of three independent cell isolations. *P<0.05, **P<0.01 comparing untreated with treated at each time point. Insert shows untreated cell data in more detail.

4.2.4 $ACTH_{1-24}$ stability studies

To examine the stability of $ACTH_{1-24}$ over the experimental time course, BAC cells were cultured as previously described. The following morning the cells were treated with various concentrations of $ACTH_{1-24}$ which had either been prepared on the day of the experiment or prepared the night before and incubated overnight at 37°C in plastic 25ml universals.

A significant decrease in the levels of cortisol secreted into the medium overlying the cells was seen when 0.01nM $ACTH_{1-24}$ was incubated overnight at 37°C compared with $ACTH_{1-24}$ prepared on the day of the experiment, ($P<0.05$). Likewise 1nM $ACTH_{1-24}$ produced a significant decrease in cortisol output when incubated overnight ($P<0.05$). Incubating the $ACTH_{1-24}$ overnight at 37°C had no effect on 10 and 100nM $ACTH_{1-24}$, which produced a comparable response to that observed with $ACTH_{1-24}$ prepared on the day of the experiment (figure 4.4).

Figure 4.5 illustrates the effect an overnight incubation at 37°C had on forskolin (10μM) and its ability to stimulate cortisol production in BAC cells. Like the higher doses of $ACTH_{1-24}$, no difference was found between forskolin made fresh on the day of the experiment and that incubated overnight.

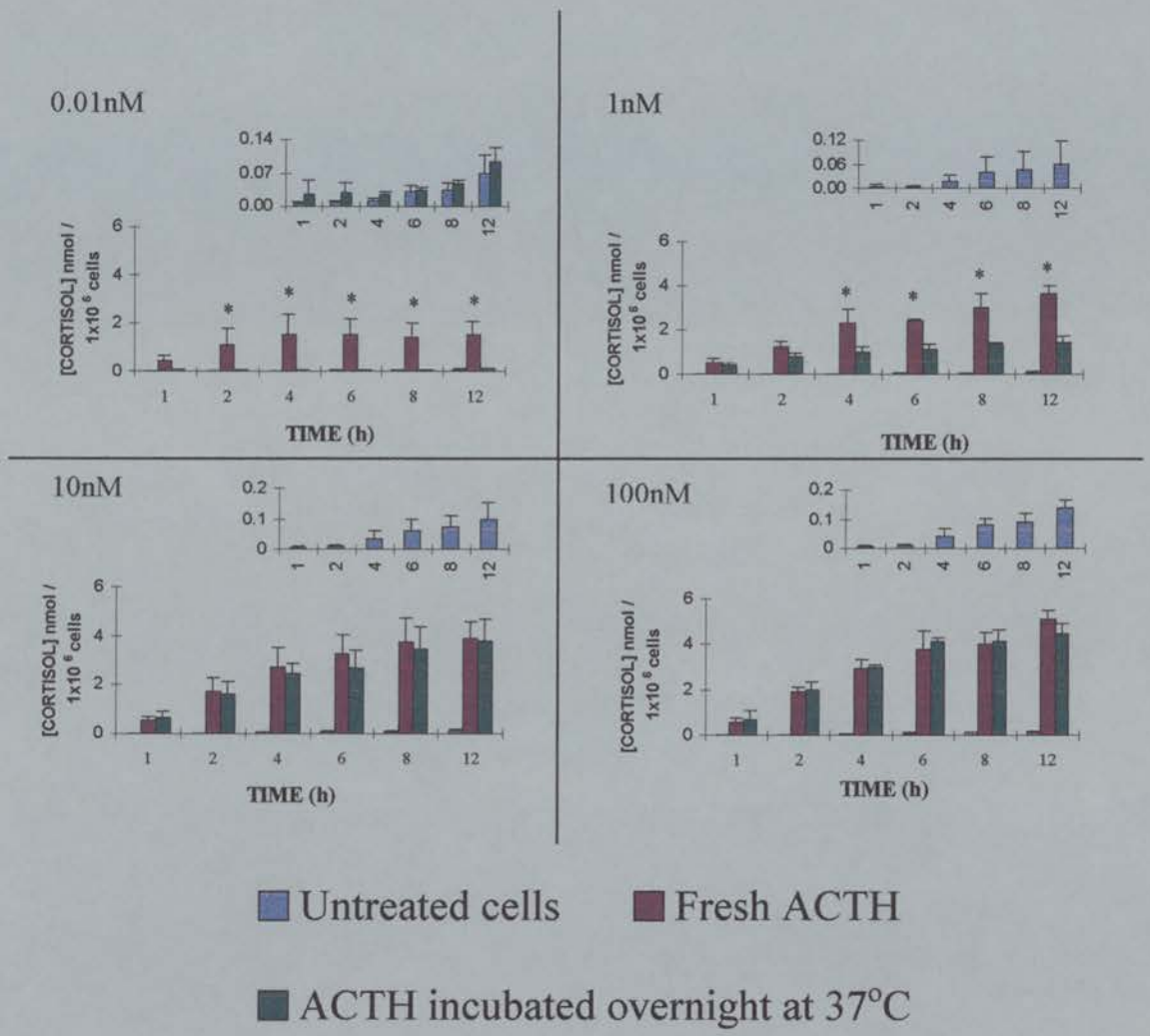


Figure 4.4 Effect of incubating $ACTH_{1-24}$ overnight at 37°C on the cortisol response by BAC cells. BAC cells were treated with or without various concentrations of $ACTH_{1-24}$ prepared on the day of the experiment (red bars) or incubated overnight at 37°C (green bars). Results are mean ± SD of three independent cell isolations. *$P<0.05$ comparing the two $ACTH_{1-24}$ conditions at each time point. Inserts display untreated cell data in more detail.

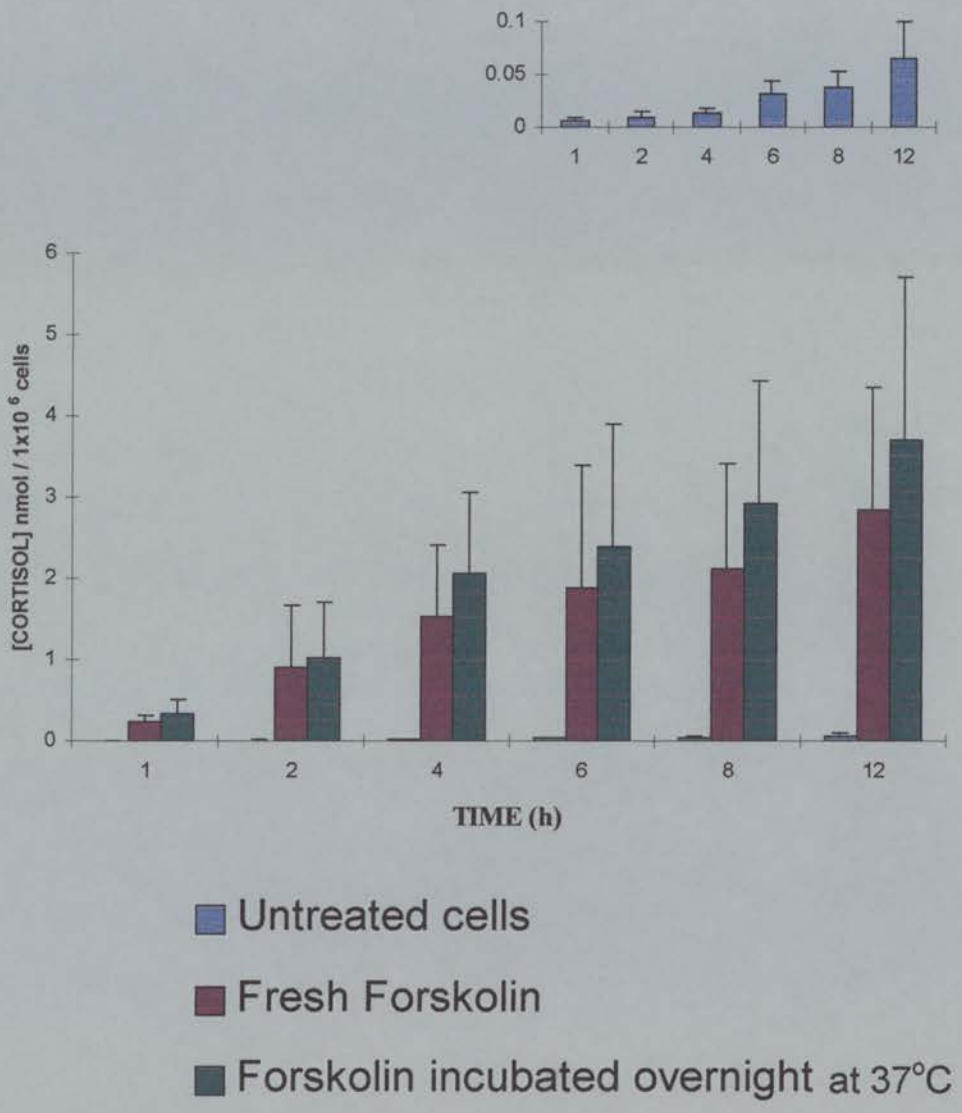


Figure 4.5 Effect of incubating forskolin (10μM) overnight at 37°C on its ability to invoke a cortisol response by BAC cells. BAC cells were treated with forskolin (10μM) prepared on the day of the experiment (maroon bars) or forskolin incubated overnight at 37°C (green bars). Results are mean ± SD of ~~three~~ one independent cell isolations.

4.2.5 $ACTH_{1-39}$ concentration in the culture medium

The next step was to measure the concentration of ACTH in the cell culture medium to determine if the ACTH concentration remained constant over the time span of the experiments. The measurement of ACTH was carried out by a two-site immunometric assay, using antibodies directed against the 18-39 C-terminal fragment and the 1-24 N-terminal fragment. Thus ensuring that only the intact $ACTH_{1-39}$ molecule is detected (see section 2.2.4). Therefore $ACTH_{1-39}$ was used in these experiments. Three concentrations were incubated in tissue culture plates overnight at 37°C. The $ACTH_{1-39}$ concentrations in the medium before and after the incubation were measured.

$ACTH_{1-39}$ concentrations of 1, 10 and 100nM were used and figure 4.6 shows that at all the concentrations there was a decrease in the $ACTH_{1-39}$ concentration measured. After an overnight incubation at 37°C the concentration of $ACTH_{1-39}$ in the medium containing 1nM decreased by 90% to 0.1nM, this value was obtained in both the experiments. The 10nM $ACTH_{1-39}$ sample decreased by 73% to 2.7nM (2.5/2.9nM in the individual experiments), and the 100nM sample decreased by 39% to 61nM (54/68nM in the individual experiments).

To determine if the decrease in $ACTH_{1-39}$ concentration was affected by the presence of BAC cells in the tissue culture plates, a second experiment was performed where $ACTH_{1-39}$ (10nM) was incubated in tissue culture plates in the presence or absence of BAC cells for 1, 6, 12 and 24 hours (Figure 4.7). In the absence of BAC cells the $ACTH_{1-39}$ concentration in the medium was reduced by 60% within the first hour, with a 77% reduction found at 6, 12 and 24 hours incubation. When compared with the original concentration of 10nM at 0 h, these reductions were found to be significant at each of the time points studied ($P<0.05$).

The presence of BAC cells in the culture plates impedes this dramatic decrease in the $ACTH_{1-39}$ concentration, with only a 3, 10, 17 and 27% reduction in concentration observed after 1, 6, 12 and 24 hours incubation respectively. No significant difference was found between 0 hour and any of the time points studied.

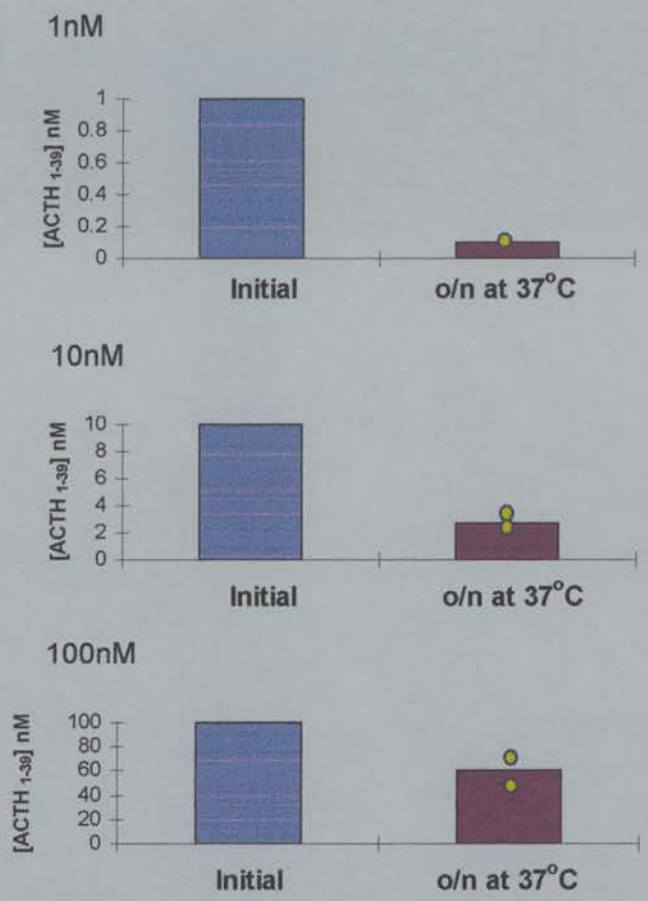


Figure 4.6 Concentration of $ACTH_{1-39}$ in the medium overlying BAC cells. Ham's F10 with 0.2% BSA containing $ACTH_{1-39}$ was incubated overnight at 37°C in tissue culture plates prior to $ACTH_{1-39}$ measurement. Results are means of two independent experiments, the yellow dots are the value for each of the experiments (1nM both values were 0.1nM; 10nM values were 2.5 and 2.9; 100nM values were 54 and 68).

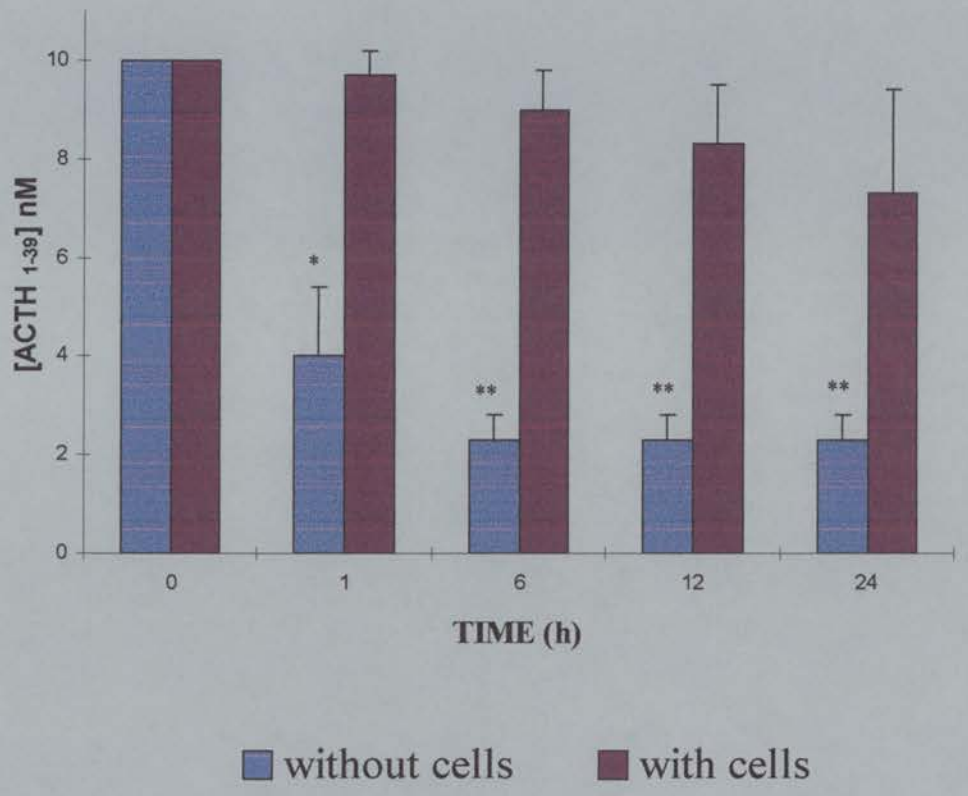


Figure 4.7. Concentration of $ACTH_{1-39}$ in the medium with or without BAC cells present. $ACTH_{1-39}$ (10nM) was incubated in tissue culture plates with or without BAC cells present as indicated. The medium was removed and assayed for $ACTH_{1-39}$ content. Results are mean ± SD of three independent measurements. *P<0.05, ** P<0.01 comparing each time point with zero hour..

4.2.6 Effects of conditioned medium

BAC cells were cultured as previously described. One set of cells (set A) were treated with or without $ACTH_{1-24}$ (10nM) for 24 hours in Ham's F10 plus 0.2% BSA. The second set of cells (set B) were serum-deprived overnight as previously described. The following morning the medium from set A cells was removed and transferred onto set B cells. Five experimental conditions were set up: 1) Normal response 2) Control conditioned medium, 3) Control conditioned medium plus $ACTH_{1-24}$ (10nM), 4) ACTH-conditioned medium, 5) $ACTH_{1-24}$-conditioned medium plus $ACTH_{1-24}$ (10nM) see below.

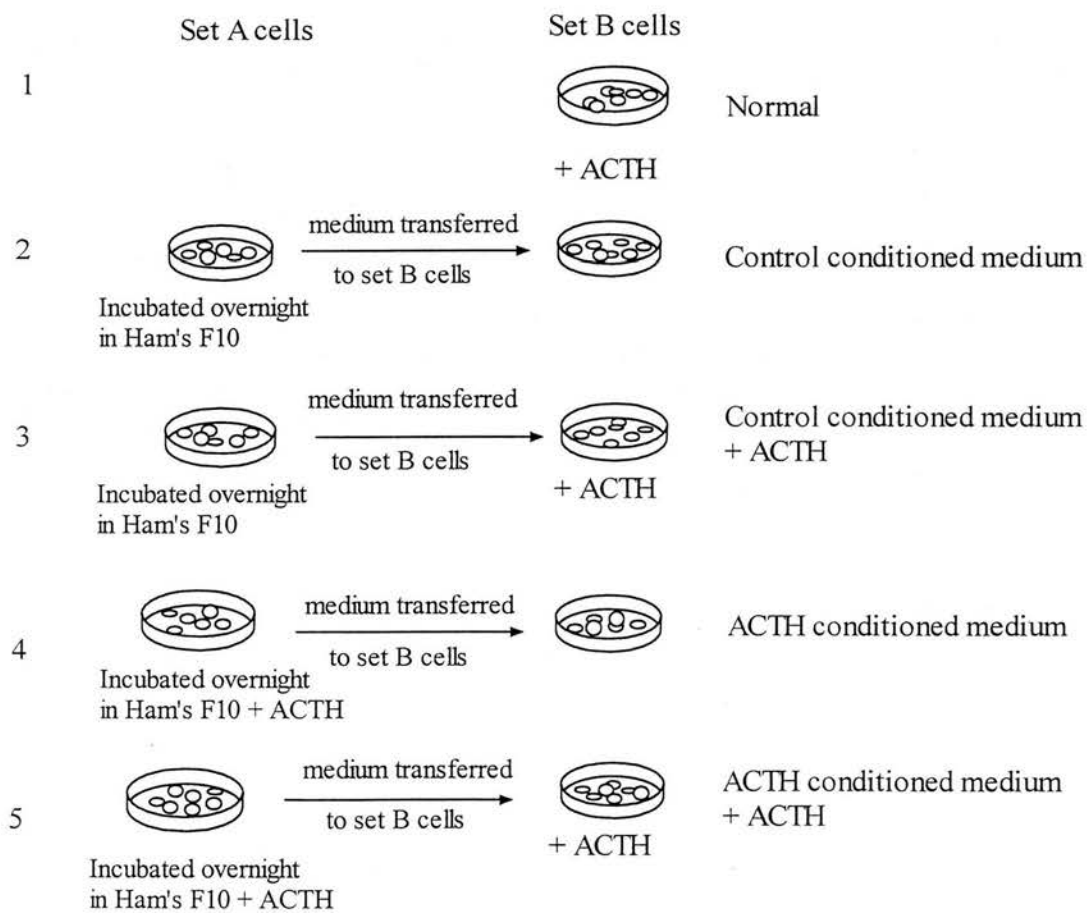


Figure 4.8 Set up for conditioned medium experiment.

Figure 4.9A illustrates the normal response of BAC cells treated with $ACTH_{1-24}$ for the times indicated. As previously reported there was an increase in the cortisol output when the cells are treated with $ACTH_{1-24}$ compared with untreated cells at all of the time points studied, $P<0.05$. With a plateau in the cortisol output occurring at around 6 hours.

Figure 4.9B illustrates the response by BAC cells incubated in control-conditioned medium with or without $ACTH_{1-24}$-treatment. $ACTH_{1-24}$-treatment significantly increased the amount of cortisol secreted into the medium overlying the BAC cells compared with untreated cells ($P<0.05$). The levels of cortisol secreted into the medium overlying the cells were higher than those obtained under normal conditions and no plateau in cortisol output was found when the data was analysed using the nonlinear curve equation.

Figure 4.9C illustrates the response by BAC cells incubated in ACTH-conditioned medium with or without $ACTH_{1-24}$-treatment. Due to the 24 hour incubation with $ACTH_{1-24}$ prior to experimentation there was already an increased amount of cortisol in the medium, 7.2 ± 0.1nmol/10^6 cells. When the $ACTH_{1-24}$-conditioned medium was placed on BAC cells there was no additional increase in the amount of cortisol secreted into the overlying medium. The addition of further $ACTH_{1-24}$ to the conditioned medium appeared to produced an increase in the amount of cortisol secreted by BAC cells into the surrounding medium; however this trend was found to be statistically insignificant. The amount of cortisol secreted into the medium overlying the cells at 8 and 12 hour was 3.9 ± 0.2 and 4.5 ± 1.2nmol/10^6 cells respectively, equivalent to the levels of cortisol found under normal conditions at 4 and 6 hours.

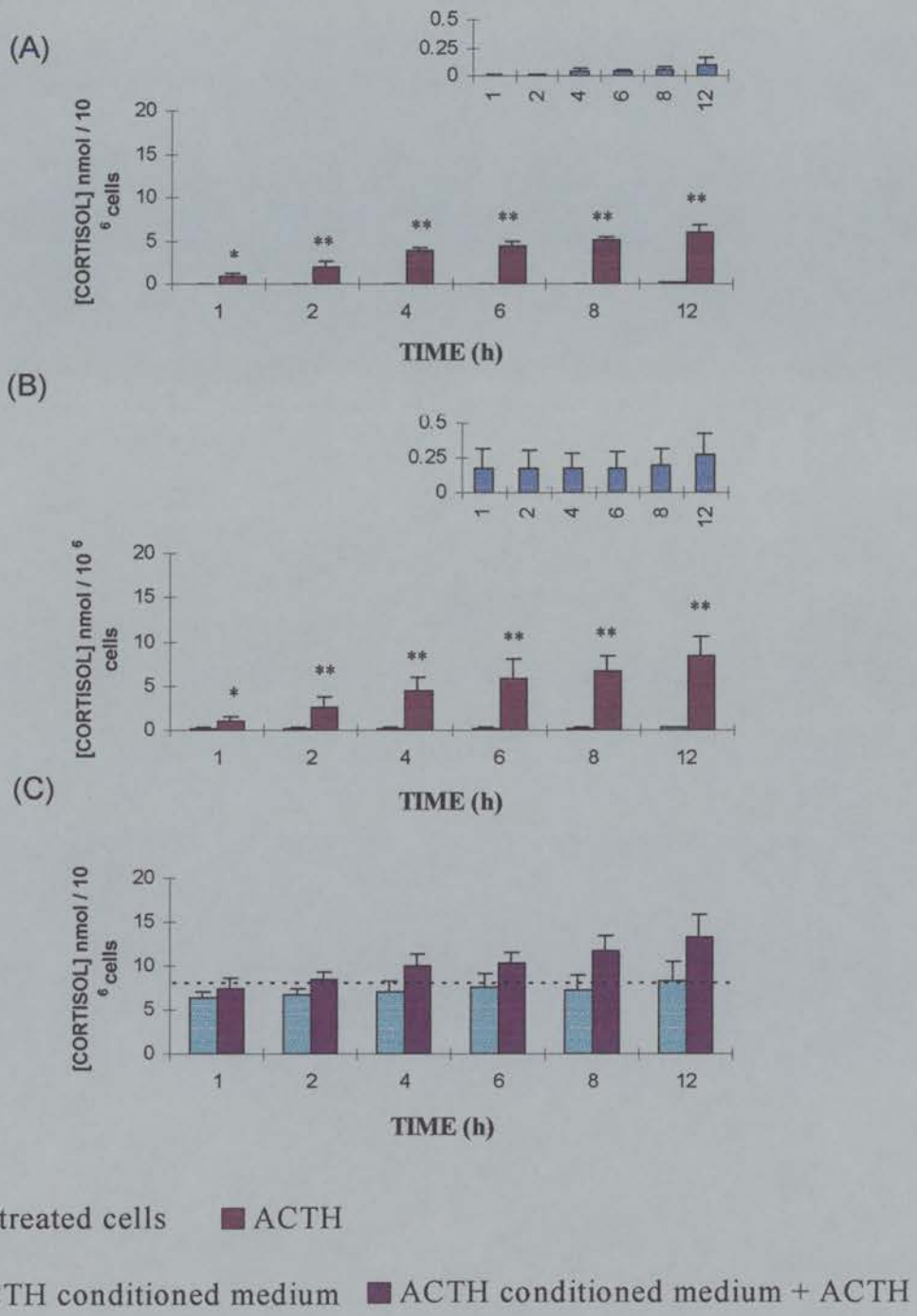


Figure 4.9 Effects of conditioned medium BAC cells were treated as A, normal; B, with control conditioned medium or C, with $ACTH_{1-24}$-conditioned medium. The medium was then removed at the times indicated and assayed for cortisol. Results shown are mean ± SD of three independent cell isolations. Inserts display untreated cell data in more detail. $P<0.05$, $**P<0.01$ comparing $ACTH_{1-24}$-treated with untreated cells at each time point studied. The dotted line indicates the level of cortisol present in the medium at 0h.

Figure 4.10 displays the various steroids secreted into the medium overlying the cells in figure 4.9C As previously determined four steroids are secreted into the medium, cortisol, corticosterone, 11β-hydroxyandrostenedione and cortisone. The cortisol values obtained by HPLC were similar to those obtained by the RIA method, 9.0 ± 4.5nmol/10^6 cells compared with 8.1 ± 1.6nmol/10^6 cells for the RIA method at 1 hour and 13.6 ± 2.8nmol/10^6 cells compared with 14.5 ± 3.6nmol/10^6 cells for RIA, at 12 hour treatment with $ACTH_{1-24}$-conditioned medium plus $ACTH_{1-24}$. As with the RIA method, HPLC analysis showed no significant difference between $ACTH_{1-24}$-conditioned medium and those treated with $ACTH_{1-24}$-conditioned medium plus $ACTH_{1-24}$ at any of the time points studied.

The amount of corticosterone in the medium after the 24 hour $ACTH_{1-24}$-treatment was 0.2 ±0.1nmol/10^6 cells. When this ACTH-conditioned medium was placed on BAC cells the corticosterone content of the medium remained constant over the 12 hour period. When $ACTH_{1-24}$ was added to the ACTH-conditioned medium the amount of corticosterone secreted into the medium increased over the initial 4 hours, declining thereafter. No significant difference was found between either treatment at any of the time points.

The amount of cortisone and 11β-hydroxyandrostenedione in the medium after the 24 hour $ACTH_{1-24}$-treatment was 1.4 ±0.3 and 0.5 ±0.1nmol/10^6 cells respectively. The levels of both steroids in the medium remained fairly constant when the BAC cells were incubated with $ACTH_{1-24}$-conditioned medium. No significant difference was observed in the cortisone or 11β-hydroxyandrostenedione levels when further $ACTH_{1-24}$ was added to the conditioned medium at any of the time points studied.

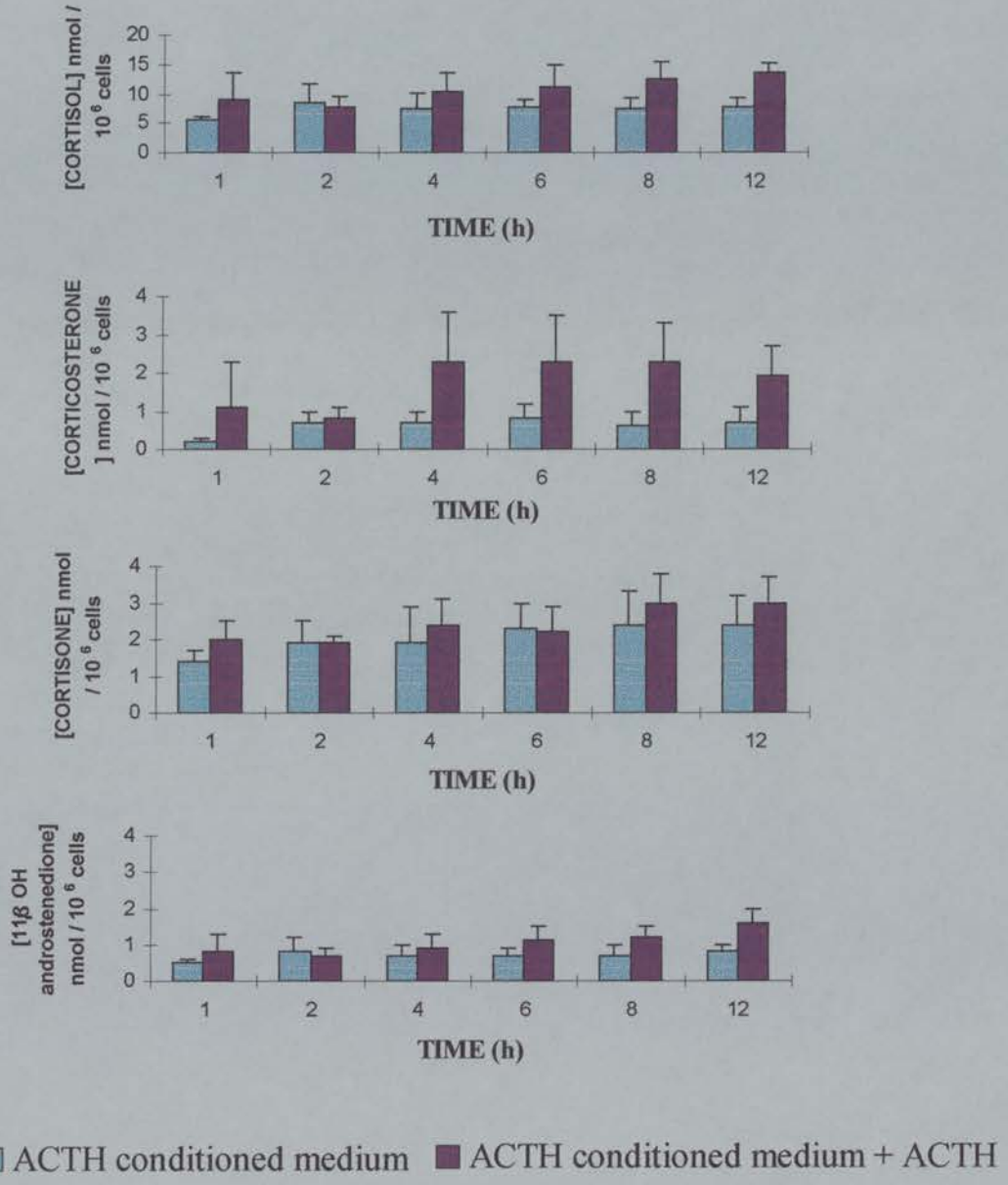


Figure 4.10 HPLC analysis of $ACTH_{1-24}$-conditioned medium BAC cells were treated with $ACTH_{1-24}$-conditioned medium, with or without $ACTH_{1-24}$. The medium was then removed at the times indicated and analysed by HPLC. Results shown are mean ± SD of three independent cell isolations.

4.3 Discussion

The hypothesis that the plateau seen in cortisol production over the 12 hour experimental time period was due to ACTH receptor desensitisation was investigated. Using BAC cells Kramer et al observed a decline in the cortisol output with time. One suggestion for this decline was ACTH receptor down regulation (i.e. a decrease in receptor number on the cell surface) (Kramer, 1983). To investigate the continued responsiveness of the cells used in this thesis, BAC cells were treated for 24 hours with ACTH, followed by a second ACTH-treatment for the 12 hour experimental period. The cortisol output in response to this second ACTH-treatment was comparable with the pattern observed in the initial experiments. Indeed the cells pre-incubated with ACTH for 24 hours produced slightly higher levels of cortisol throughout the time course, which was significant at the later time points of 8 and 12 hours. Also the plateau in cortisol output occurred later than in non pre-treated cells. The reason for this improved cortisol response with 24 hours ACTH pre-treatment was most likely due to the up-regulation in the activity of the steroidogenic enzymes. A study by Jefcoate et al concluded that the decline in cortisol response, by BAC cells, to ACTH treatment was not the result of a loss of sensitivity of the cells to ACTH, as the 17α-hydroxylase activity increased rapidly between 12 and 24 hours following ACTH addition (DiBartolomeis, 1984). Another study using cultured bovine adrenal cells showed that ACTH was one of the few trophic hormones to have a positive effect on its own receptor and that the binding of ACTH to cells was lower on day 1 than on day 2 of culture, remaining fairly constant for a further 2 days. This could be due to a slow recovery of membrane-bound receptors after cell isolation by enzymatic digestion (Penhoat, 1989). ACTH increased ACTH-receptor mRNA, in a time- and dose-dependent manner, (Penhoat, 1994) and ACTH receptor number (Penhoat, 1995).

The continued responsiveness of the ACTH receptor was also demonstrated. BAC cells were shown to respond to renewed ACTH treatment every 12 hours through 72 hours. The cortisol output remained reasonably constant throughout the period of study. These results indicate that in BAC cells the ACTH receptor does not become desensitised but remains fully functional to long-term ACTH-treatment. The conundrum concerning an adequate supply of cholesterol is also answered by these results. For the cells to continue responding to ACTH-treatment for 72 hours there must be sufficient cholesterol available to sustain steroidogenesis. Rainey et al measured the total cholesterol content of BAC cells under lipoprotein-deplete conditions, treating the cells every 12 hours through 48 hours and found that the total cholesterol content of the cells decreased by 20% after the first 12 hours of ACTH treatment with no further decrease thereafter (Rainey, 1986).

This chapter also explored some aspects of the cell culture system. Primary culture is a foreign environment for BAC cells, lacking the structural morphology and the biochemical and nervous mechanisms pertaining to the adrenal. The removal from such a controlled situation may be expected to have some affects on the functional characteristics of the cells. Nevertheless, the cells respond to ACTH, by producing cortisol, suggesting that BAC cells in culture are largely representative of adrenocortical cells *in vivo*.

A basic requirement for the well being of cells in culture is the availability of a range of nutrients. There are various media used to culture BAC cells. Ham's F10 is the medium used by this laboratory, though another medium of choice by a number of researchers is DMEM/F12. Both media have a similar amino acid and vitamin composition but DMEM/F12 contains 3g/l D-glucose, three time the amount found in Ham's F10. To discover if the glucose concentration had any effect on the cortisol response, BAC cells were maintained in both media. The increase in glucose concentration did not alter the trend in cortisol output from BAC cells treated with

$ACTH_{1-24}$ over a 12 hour period. When the glucose concentration was measured in the Ham's F10 medium overlying the cells it was found not to have decreased significantly over the 12 hours. This implies that Ham's F10, as far as glucose concentration is concerned, was an adequate medium for culturing BAC cells and did not play a part in the decline in cortisol output observed.

The stability of $ACTH_{1-24}$ in the culture system was studied next. In order to resolve this problem, $ACTH_{1-24}$ was incubated, in tissue culture dishes, overnight at 37°C prior to treatment on BAC cells. The response of $ACTH_{1-24}$ (100 and 10nM) was comparable to the response seen with freshly prepared $ACTH_{1-24}$ at these concentrations, indicating that there was no deterioration in ACTH stability. This was not the case with lower doses of $ACTH_{1-24}$. When $ACTH_{1-24}$ at concentrations of 1 and 0.01nM were incubated overnight at 37°C prior to placing on the cells a significant decrease in the cortisol response was seen. These results suggest the bioactivity of ACTH is affected when utilised in the cell culture system. ACTH in the medium was measured to investigate what effect, if any, overnight incubation at 37°C was having on its concentration. Because the measurement of ACTH was carried out using a 2-site immunometric assay (see 2.2.4), it was necessary to use $ACTH_{1-39}$ for these experiments. The concentration of $ACTH_{1-39}$ was found to decrease at all of the concentrations measured with the largest decrease seen with the lower concentrations. This decrease in the $ACTH_{1-39}$ concentration was less marked at 10nM when BAC cells were present in the tissue culture plates. It is well known that ACTH and other peptide hormones bind to plastic; however, no reference pertaining to this phenomenon was found. The results in figure 4.12 support this hypothesis. In the absence of BAC cells there was a larger surface of plastic for the $ACTH_{1-39}$ to bind; however when cells were present the $ACTH_{1-39}$ comes into contact with less area of plastic and therefore binding was reduced.

The binding of ACTH to the plastic of the tissue culture plates partly explains the reason for the plateau in cortisol output by BAC cells. The reduction in bioactivity of ACTH at 10nM was less marked in the presence of BAC cells.

An alternative explanation for the plateau in cortisol output is that some factor(s) was secreted into the cell culture medium preventing further steroidogenesis. This element would be removed *in vivo* via the blood system; however, this would not be possible within the BAC cell culture situation leading to the accumulation of this factor(s) and the inhibition of further steroidogenesis. This theory was tested using 'conditioned medium' (section 4.2.6). BAC cells incubated with control-conditioned medium plus ACTH-treatment displayed a significant increase in cortisol output compared with cell incubated in control-conditioned medium alone at each of the time points studied. The levels of cortisol produced by cells incubated with control-conditioned medium, with or without ACTH-treatment, were found to be higher than those obtained under normal conditions. An interesting observation in the control-conditioned medium cells treated with ACTH was that no plateau in the cortisol output was observed. The presence of some factor secreted by BAC cells into the control-conditioned medium appears to augment the ACTH-induced response.

After 24 hour of ACTH-treatment cortisol, cortisone, corticosterone and 11β-hydroxyandrostenedione were found in the medium overlying BAC cells. When this medium is placed on cells which had not been treated with ACTH no further synthesis of steroids was found. This suggests the presence of one or more factors in the medium preventing further steroidogenesis or as previously shown, the bioactivity of ACTH was reduced over the 24 hour incubation. Upon the addition of a second ACTH-treatment there was a statistically insignificant trend for an increase in cortisol secretion over the time course. These data suggest the presence of a factor, possibly cortisol or another steroid, in the ACTH-conditioned medium which

attenuate the steroidogenic response by BAC cells, but which may be degraded overtime.

Glucocorticoids have a diverse array of actions which are mediated by the glucocorticoid receptor (GR). Among the factors that alter the levels of GR expression, glucocorticoids themselves appear to be the most potent regulators and have been shown to down-regulate the receptor in some cell lines (Burnstein, 1991), (Burnstein, 1992). It is conceivable, that in BAC cells a local negative feedback loop exists whereby excess cortisol causes a down-regulation of GR expression and thereby preventing further steroidogenesis.

In summary. the decline in cortisol production was found not to be the result of ACTH receptor desensitisation. The binding of ACTH to the plastic of the tissue culture plates has been shown to be a problem, although this was reduced by the presence of cells. The secretion of a some factor or factors into the medium overlying the cells may have some effect on the steroidogenic ability of the cells, possibly acting via the GR. The complex nature of cell culture medium and the components secreted into it by the cells make this line of investigation difficult.

The results presented in this chapter have provided some possible explanations for the plateau in cortisol production found in ACTH-treated BAC cells. Further studies to investigate the effect of ACTH on other aspects of steroidogenesis, such as StAR protein, may help provide some answers.

CHAPTER 5 : EXPRESSION OF STEROIDOGENIC ACUTE REGULATORY (StAR) mRNA IN CULTURED BAC CELLS.

5.1 Introduction :

The rate-limiting enzymatic step of steroidogenesis is the conversion of cholesterol to pregnenolone by CYP11A which is situated on the inner mitochondrial membrane. However, the acutely regulated step in steroid production is the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (as discussed in chapter 1). In rat adrenal this acute step was shown to involve the production of a family of 28-to 30kDa proteins (Krueger, 1983). Using techniques such as [^{35}S] methionine/cysteine-labeling and 2-D gel electrophoresis it was shown that bovine adrenal glomerulosa and fasciculata cells responded to steroidogenic stimuli with the rapid appearance of a family of 28- to 30kDa proteins similar to those described in the rat by Krueger (Elliott, 1993), (Hartigan, 1995). The family consists of a phosphorylated form found in ACTH-treated cells and an unphosphorylated form in untreated cells. The 30kDa protein, later named StAR protein, is synthesised as a larger cytosolic precursor protein of approximately 37kDa. The 37kDa protein is targeted to the mitochondria where it is processed to its mature 30kDa form (Epstein, 1991b), (Stocco, 1991), (Hartigan, 1995). The 37kDa protein is rapidly processed proteolytically to the 30kDa protein, which accumulates in the mitochondria of steroidogenic cells (Hartigan, 1995), (Stocco, 1991).

StAR protein was purified and the cDNA cloned and sequenced in mouse Leydig tumor MA-10 cells. The bovine StAR cDNA was cloned from differential cDNA expression studies of corpora lutea cells in the late stages of the estrous cycle. The bovine cDNA sequence was found to be 84% homologous with the transcripts for the human and the mouse steroidogenic acute regulatory protein (Hartung, 1995).

Comparison of bovine tissue showed that the expression of StAR mRNA was strongest in ovarian corpora lutea and adrenal. All other tissues studied including heart, lung, pituitary and liver showed no detectable StAR mRNA expression. Surprisingly no StAR mRNA expression was found in the testis (Hartung, 1995), which has been shown to express StAR mRNA in humans (Sugawara, 1995a). A later study did show the expression of StAR mRNA in bovine fetal and adult testes (Pilon, 1997), the reason for this discrepancy is unclear. Although StAR protein is not expressed in human placenta it has been found in the placenta of cow (Pescador, 1996), (Pilon, 1997). Immunohistochemical staining has demonstrated the presence of StAR protein in the zona glomerulosa, fasciculata and reticularis of the bovine adrenal (Lo, 1998).

Analysis of the StAR mRNA in the bovine adrenal reveals that there are two specific transcripts, 3.0 and 1.8 kilobases (kb) (although a minor transcript at 1.6kb is sometimes seen) (Hartung, 1995). Studies on other species have shown three transcripts in the mouse (Clark, 1995b); human (Sugawara, 1995a); rat (Lee, 1997) and pig (Pescador, 1997) and one transcript in sheep (Juengel, 1995). The functional significance of the different sizes of transcripts is as yet unknown; however, it is thought that the differences in length maybe due to differences in length of the 3' untranslated regions (Hartung, 1995).

It has been demonstrated that there is a close temporal relationship between steroid biosynthesis and StAR mRNA and protein synthesis (Clark, 1995b). In mouse Leydig tumor MA-10 cells StAR mRNA and protein have been shown to be induced in close coordination via a cAMP-mediated mechanism, within a time frame concomitant with the acute production of steroid hormones (Clark, 1995b). The continued translation of StAR protein and steroidogenesis has been shown to be dependent on the continued transcription of the StAR gene (Clark, 1997).

The aims of this chapter were to establish the appropriate culture conditions with which to study the expression of StAR mRNA in BAC cells. The results presented in this thesis thus far have demonstrated that BAC cells show an enhanced responsiveness to ACTH-treatment on day 3 of culture. The use of the culture medium Ham's F10 and serum-free conditions were also shown to be appropriate for the study of cortisol secretion in BAC cells. To investigate whether the expression of StAR mRNA by ACTH-treated BAC cells in culture paralleled the cortisol response, experiments similar to those performed for cortisol production were carried out.

Once the culture conditions were established, the expression of the StAR transcripts in response to ACTH and other agonists was studied.

5.2 Results :

5.2.1 Expression of StAR mRNA in $ACTH_{1-24}$-treated BAC cells with time in culture.

BAC cells were treated with $ACTH_{1-24}$ (10nM) on the day of isolation (day 0), and on consecutive days in culture through day 4. The cells were cultured in $25cm^2$ tissue culture flasks with $5x10^6$ cells/flask. The cells on day 0 were treated in sterile 25ml universals ($5x10^6$ cells/universal). The cells were maintained in Ham's F10 containing 10% CPSR1 throughout the experiment, see 3.2.1 for more details on experimental protocol. On each day the cells were treated with or without $ACTH_{1-24}$ (10nM) for 6 or 24 hours. The time points were chosen as the literature available at the time showed the expression of StAR mRNA in the MA-10 mouse Leydig tumor cell line was maximal at 6 hours of dbcAMP-treatment followed by a decline in levels (Clark, 1995).

The quantification data for StAR mRNA is presented in three ways; 1) StAR mRNA levels as quantified by the phosphoimaging system, the results are the sum of both the 3.0 and 1.8kb transcripts. This was to take into account any variability between the two transcripts. 2) The StAR mRNA levels corrected for actin, statistics were performed on the actin corrected graph only. Actin was used to ensure equal loading of the RNA onto the agarose gel. Therefore, any change in the StAR transcript levels was due to the treatment of the cells and not unequal amounts of RNA loaded onto the gel. 3) Inserts show the appropriate data relative to the untreated levels. This takes into account any variability in the untreated levels over the experimental time course.

Figure 5.1 displays the northern hybridisation of StAR mRNA expression by BAC cells. Consistent with other studies, two StAR transcripts were found, 3.0 and 1.8kb. The expression of StAR mRNA, at both the 6 and 24 hour time points, was found to be highest on the day of isolation (day 0). The expression of the StAR transcripts was raised in both the untreated and the $ACTH_{1-24}$-treated cells on day 0. On day 1 of culture the expression of the StAR transcripts had fallen. The expression of the transcripts in the untreated cells was undetected and an induction of the StAR transcripts in response to $ACTH_{1-24}$-treatment was observed at 6 and 24 hours. A similar pattern was found on day 2 of culture. The StAR mRNA expression by BAC cells in response to $ACTH_{1-24}$-treatment for 6 hours was found to be maximal on day 3 of culture. However, the expression of the StAR transcripts, in response to $ACTH_{1-24}$-treatment, had fallen to untreated levels by 24 hours on day 3. A similar pattern was seen on day 4.

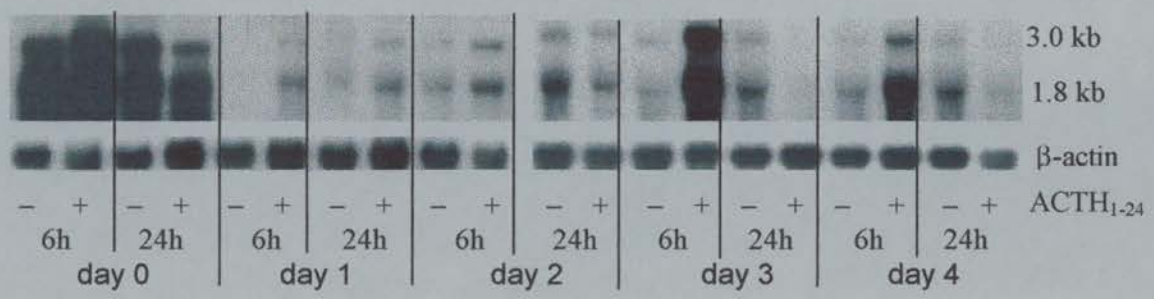


Figure 5.1 Representative northern hybridisation of StAR mRNA expression in BAC cells treated on separate days of culture. BAC cells were treated with or without $ACTH_{1-24}$ as indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. Two transcripts were found; 3.0 and 1.8 kb. The membrane was stripped and reprobed for β-actin to ensure equal loading of RNA.

Figure 5.2 displays the quantified levels of the two StAR transcripts. Due to the high levels of StAR mRNA found on day 0 were there was no significant difference in the StAR mRNA levels between the untreated cells and the $ACTH_{1-24}$-treated cells at either of the time points studied. On each of the other days in culture a significant increase in the amount of StAR transcript levels in $ACTH_{1-24}$-treated cells compared with untreated cells at 6 hours was seen ($P<0.05$). No significant difference was seen between untreated and $ACTH_{1-24}$-treated cells at 24 hours on any of the days studied.

The levels of the StAR transcript was found to be significantly lower, for both untreated and $ACTH_{1-24}$-treated cells, on days 1 and 2 compared with day 0 ($P<0.05$). The expression of StAR mRNA in $ACTH_{1-24}$-treated BAC cells on day 3 is significantly greater than that found on day 1 ($P<0.05$). Day 3 of culture also displays a 9-fold increase in StAR mRNA expression at 6 hours by $ACTH_{1-24}$-treated BAC cells when compared with untreated cells, $P<0.05$. However there is no significant difference between untreated and treated values at 24 hours. Again on day 4 the trend was for the 6 hour $ACTH_{1-24}$-treated BAC cells to be increased over untreated cells, 3-fold increase ($P<0.05$), with no significant difference found between the two conditions at 24 hours $ACTH_{1-24}$-treatment.

It would appear from the northern analysis that the levels of StAR transcripts decreased at 24 hours on each of the day studied. The quantification of the StAR transcripts showed that this decrease was found to be significant on day 0 only ($P<0.05$). The data normalised to the untreated values (inserts) show significantly higher values at 6 hour treatments compared with 24 hours on day 1, 2 and 3 ($P<0.05$).

These results indicate that day 3 of culture was the optimum time to study StAR mRNA expression in BAC cells. This is in keeping with the cortisol data presented in chapter 3 and further experiments to study the expression of StAR mRNA were carried out on day 3.

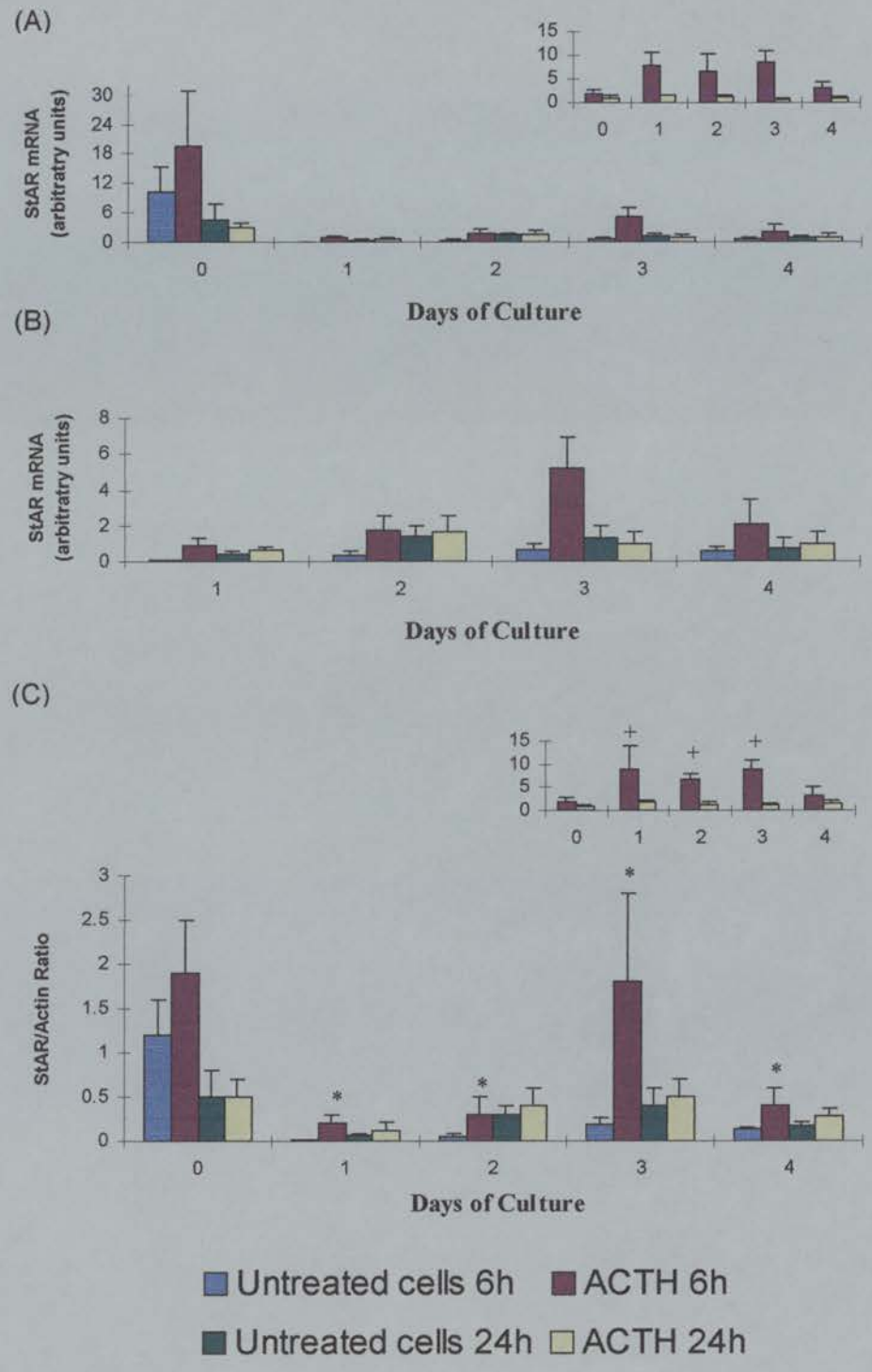


Figure 5.2 Quantification of northern hybridisation showing StAR mRNA expression in BAC cells treated on separate days of culture. A, sum of the two StAR transcripts shown in figure 5.1; B, days 1 through 4 and C, the sum of the two StAR transcripts corrected for actin. Mean ± SD from three independent cell isolations. Inserts shown values normalised to untreated cells. *P<0.05 comparing untreated and $ACTH_{1-24}$-treated. +P<0.05 comparing 6 and 24 hour $ACTH_{1-24}$-treated cells on each of the days studied.

5.2.2 Effects of serum on StAR mRNA expression

BAC cells were cultured for 2 days in 25cm^2 flasks. One set of cells was serum-deprived overnight in Ham's F10 with 0.2% BSA, the other set of cells remained in the serum-containing medium. The following day the cells were treated with $ACTH_{1-24}$ (10nM) for various times up to 24 hours.

The results indicate that StAR mRNA expression is not affected by the absence of serum (figure 5.3), akin to the cortisol data presented in chapter 3. From the northern hybridisation the expression of StAR mRNA by BAC cells, in response to $ACTH_{1-24}$-treatment, appears to be similar in both serum-free and serum-containing media. When comparing the data obtained from the phosphoimaging system, the values for the serum-free group of cells were slightly higher than those of the serum-replete; however, no significant difference was found between the two conditions at any of the time points studied. Untreated samples were analysed only at 6 hours; therefore comparisons between untreated and $ACTH_{1-24}$-treated were not possible except at 6 hours. In light of these results further experiments were carried out in serum-free medium.

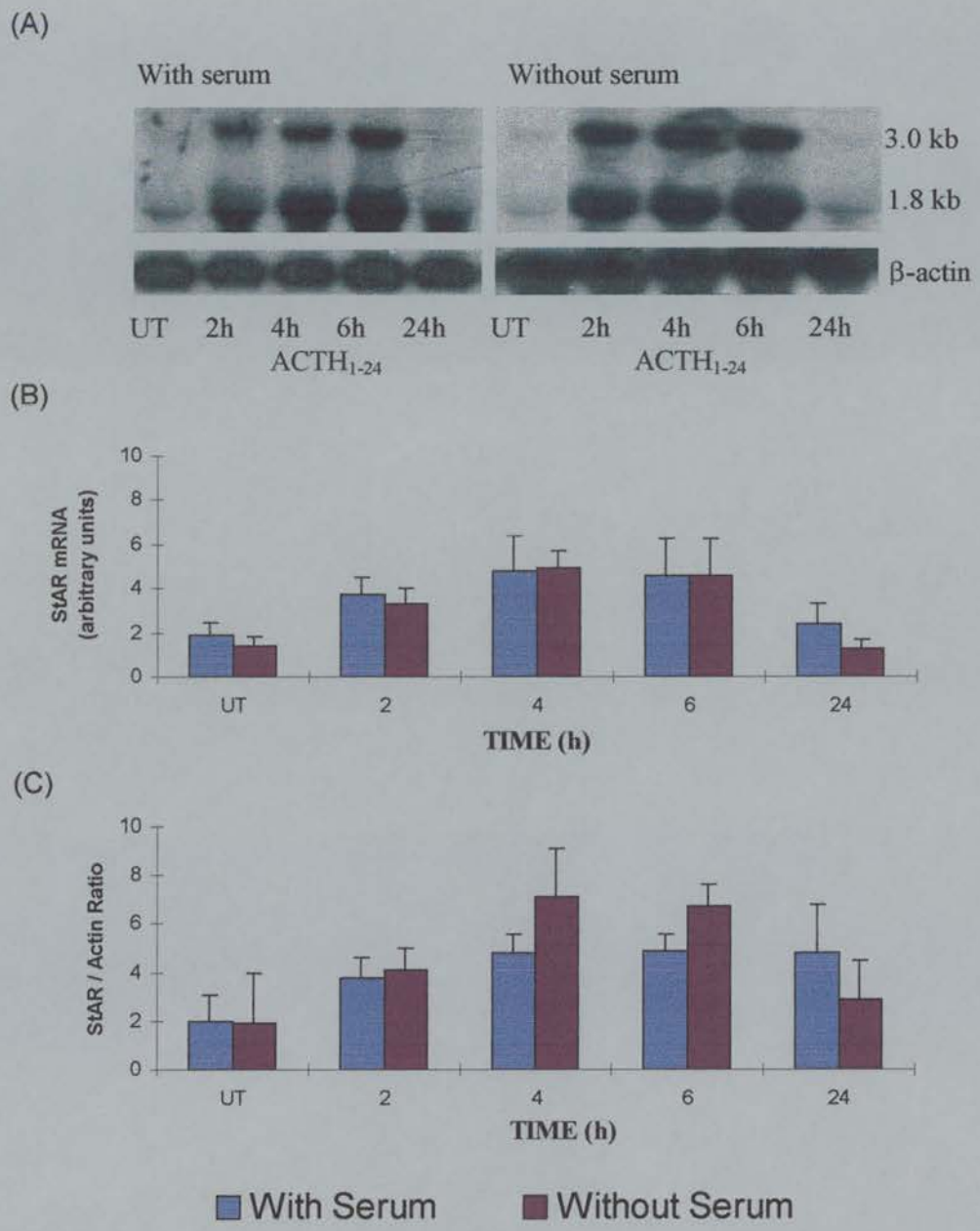


Figure 5.3 Effects of serum on StAR mRNA expression in BAC cells. BAC cells were treated with or without $ACTH_{1-24}$ (10nM), in the presence or absence of serum, as indicated, untreated (UT) sample at 6 hours only. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A, representative northern showing the two StAR transcripts at 3.0 and 1.8 kb. The membrane was stripped and reprobed for β-actin. B, quantification (sum of the two transcripts) of northern hybridisation and C, the sum of the two StAR transcripts corrected for actin. Both graphs are mean ± SD from three independent cell isolations.

5.2.3 Effect of various doses of $ACTH_{1-24}$ on the StAR mRNA expression produced by BAC cells

Figure 5.4A displays a representative northern hybridisations of StAR mRNA from BAC cells treated with $ACTH_{1-24}$ over the concentration range, 0.01pM-10nM, for 6 hours. The expression of the StAR transcripts (1.8 and 3.0kb) were increased over untreated cells, at the three highest concentrations of $ACTH_{1-24,}$ 0.1nM, 1nM, and 10nM. There did not appear to be a difference in the intensities of the bands at the two lower concentrations, 0.01pM and 1pM, when compared with the untreated bands.

Figure 5.4B&C displays the quantified data. No significant difference was found between the levels of the StAR transcript in untreated cells and those treated with $ACTH_{1-24}$ at 0.01pM and 1pM. A 2-fold increase compared with untreated cells was found at both 0.1 and 1nM $ACTH_{1-24}$ and a 3-fold increase over untreated was seen with 10nM $ACTH_{1-24}$. A significant difference in treated and untreated values was found at each of these concentrations, ($P<0.01$).

The cortisol response by BAC cells treated with various concentrations of $ACTH_{1-24}$ is displayed in figure 5.5. As with the StAR transcripts levels there is no significant increase in the cortisol output with 0.01pM $ACTH_{1-24}$; however, there was a significant increase in the amount of cortisol secreted into the overlying medium with 1pM $ACTH_{1-24}$. Again in parallel with the StAR mRNA data, there was a significant increase in the cortisol secreted into the medium overlying the cells at 0.1, 1, and 10nM $ACTH_{1-24}$.

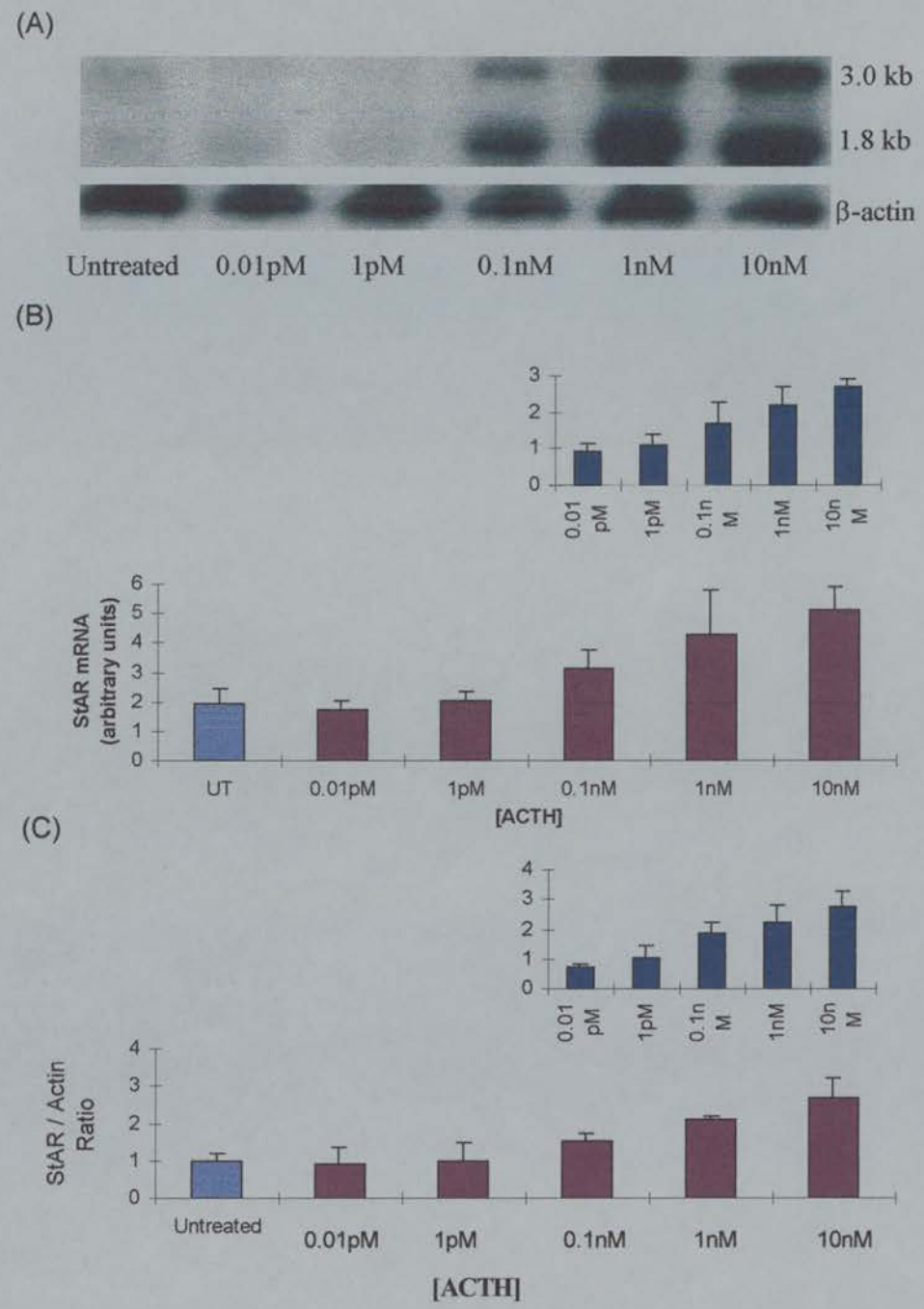


Figure 5.4 StAR mRNA levels in response to increasing doses of $ACTH_{1-24}$. BAC cells were treated with or without various doses of $ACTH_{1-24}$ as indicated for 6hours. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A, representative northern showing the two StAR transcripts, 3.0 and 1.8 kb. B, quantification of northern hybridisation shown is the sum of the two transcripts and C, corrected for β-actin. Both graphs are mean ± SD from three independent cell isolations. The inserts display the data normalised to untreated.

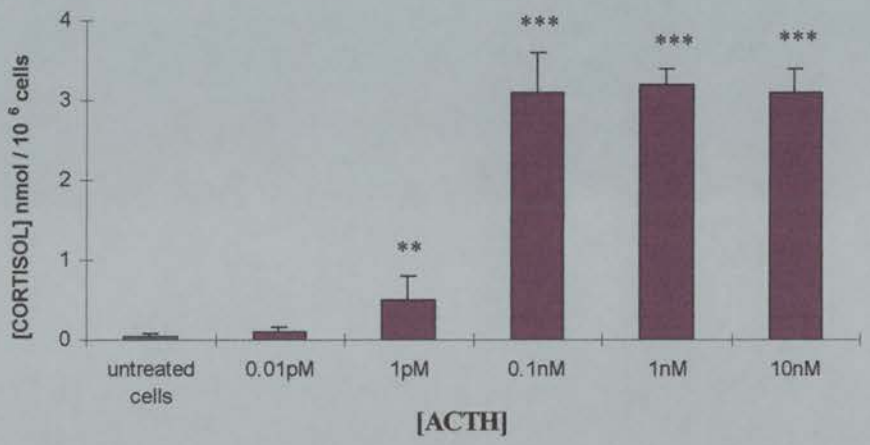


Figure 5.5 Cortisol response to various doses of $ACTH_{1-24}$. BAC cells were treated with or without various doses of $ACTH_{1-24}$ as indicated for 6hours. Results shown are mean ± SD from three independent cell isolations. **P<0.01, ***P<0.001, comparing $ACTH_{1-24}$-treated with untreated cells.

5.2.4 Expression of StAR mRNA in BAC cells in response to $ACTH_{1-24}$ at various times over a 12 hour period.

The following three figures (5.6-5.8) display the StAR mRNA expression in BAC cells treated with 1, 10 and 100nM $ACTH_{1-24}$. The representative northern hybridisations showed the two StAR transcripts, 3.0 and 1.8 kb, at all three concentrations.

Figure 5.6 shows an induction in the expression of the StAR transcripts was found after 1 hour of 1nM $ACTH_{1-24}$-treatment. The expression of the StAR transcripts increased over the initial time points, reaching a peak at between 4+6 hours and decreasing to almost untreated levels by 12 hours. Quantification of the northern confirmed these observations (figure 5.6B&C). BAC cells treated with 1nM $ACTH_{1-24}$ showed detectable StAR mRNA levels after 1 hour of treatment, which was found to be significantly different from the levels of the StAR transcripts found in untreated cells at 1 hour ($P<0.01$). There was also a significant increase in StAR mRNA levels in $ACTH_{1-24}$-treated cells compared with untreated cells at 2, 4 and 6 hours ($P<0.05$). StAR transcript levels increased over the time period, peaking at 4 hours $ACTH_{1-24}$-treatment, with a 8-fold increase compared with untreated cells found, and decreasing thereafter to almost untreated levels at 12 hour.

Figure 5.7 displays the StAR transcript expression by BAC cells treated with 10nM $ACTH_{1-24}$. Again expression of the StAR transcripts were detected after 1 hour of $ACTH_{1-24}$-treatment, with an increase in expression found at 4, 6 and 8 hours of $ACTH_{1-24}$-treatment followed by a decline in the level of the transcripts at 12 hours. The quantification data (figure 5.7B&C) illustrate a significant increase in the StAR transcript levels of 10nM $ACTH_{1-24}$-treated BAC cells at 1, 2, 4 and 6 hours ($P<0.05$). The levels of the StAR transcripts increased with time, peaking at 6 hours followed by a fall in levels at 12 hours.

The expression of the StAR transcripts by BAC cells in response to 100nM $ACTH_{1-24}$ is shown in figure 5.8. The levels of the transcripts were again detected after 1 hour of treatment, with a peak around 6 hours and a subsequent decrease in expression. Quantification of the northern hybridisation showed an increase in StAR transcript levels at all of the time points studied, with a significant increase compared with untreated cells found at 1, 2, 6, and 8 hours ($P<0.05$) (figure 5.8C). The peak in StAR transcripts was again found at 6 hours of $ACTH_{1-24}$-treatment with a 21-fold increase over untreated levels found, the levels declining thereafter.

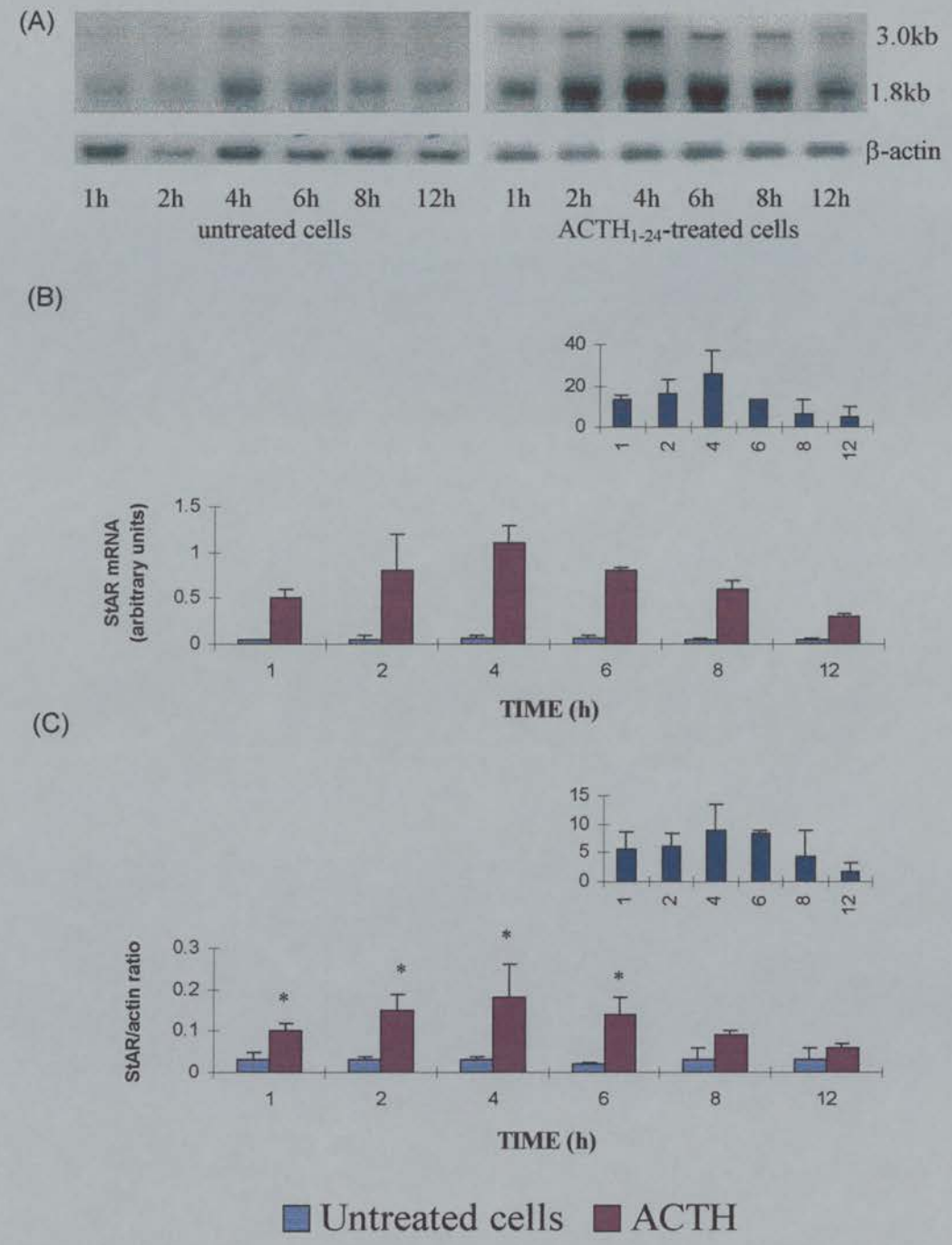


Figure 5.6. StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ (1nM). BAC cells were treated with or without $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern showing the two StAR transcripts 3.0 and 1.8kb. the membrane was stripped and reprobed for β-actin. B: quantification of northern hybridisation shown is the sum of the two StAR transcripts and C: corrected for β-actin. Both graphs are mean ± SD from three independent cell isolations. Inserts show $ACTH_{1-24}$-treated levels normalised to untreated levels. *$P<0.05$ comparing untreated and $ACTH_{1-24}$-treated levels.

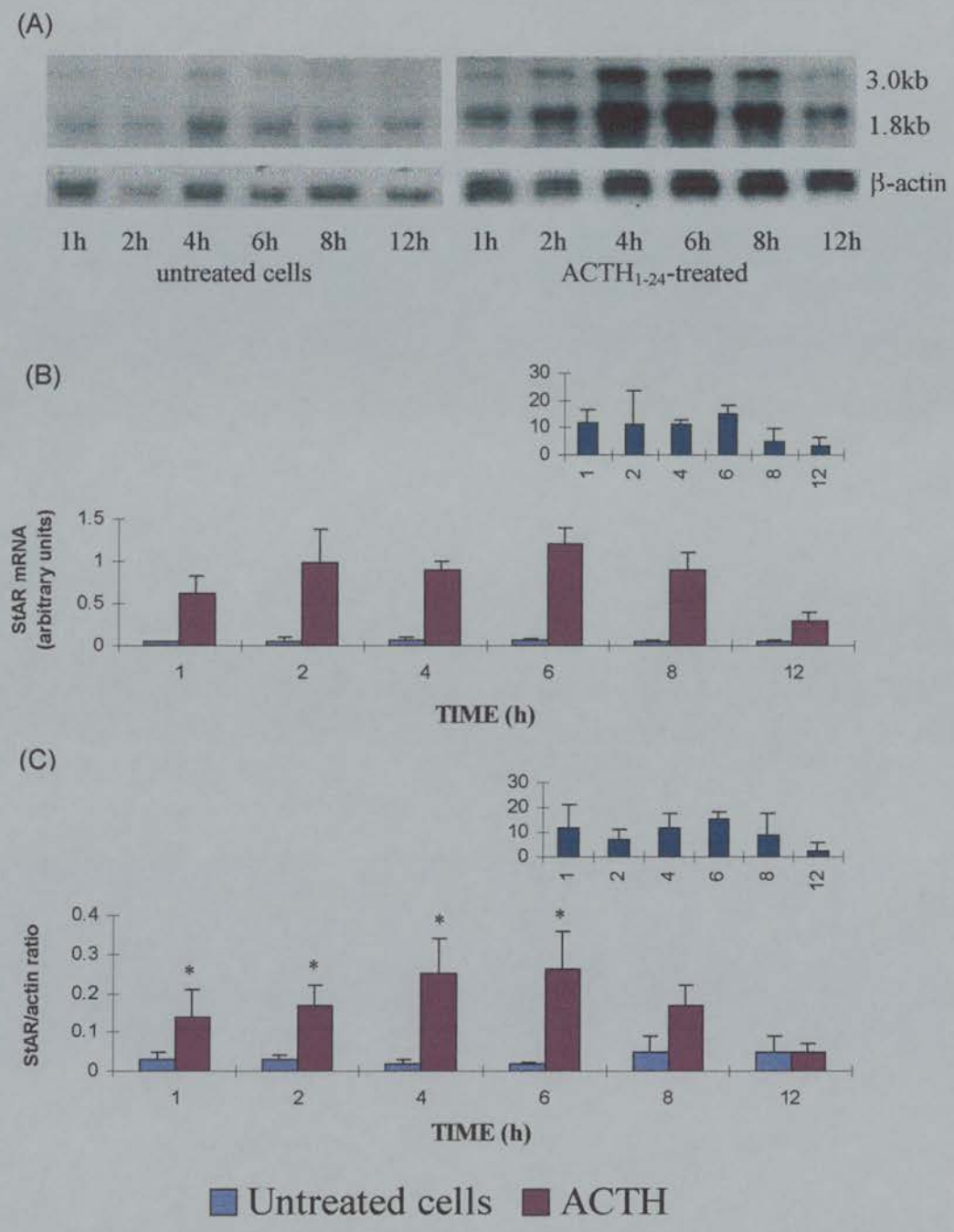


Figure 5.7. StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ (10nM). BAC cells were treated with or without $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern showing the two StAR transcripts, 3.0 and 1.8kb. The membrane was stripped and reprobed for β-actin. B: quantification of northern hybridisation shown is the sum of the two StAR transcripts and C: corrected for β-actin. Both graphs are mean ± SD from three independent cell isolations. Inserts show $ACTH_{1-24}$-treated levels normalised to untreated levels. *$P<0.05$ comparing untreated with $ACTH_{1-24}$-treated levels.

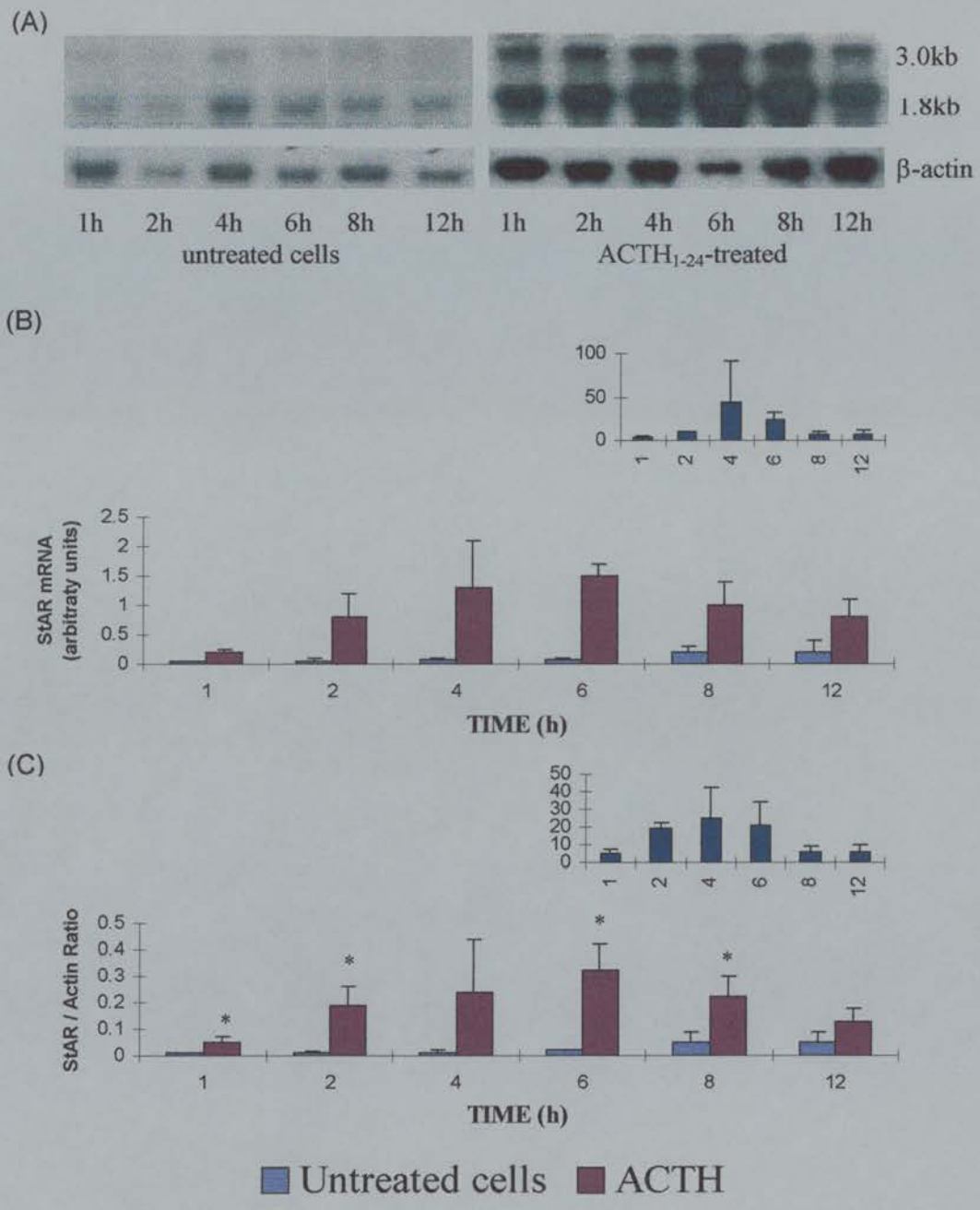


Figure 5.8. StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ (100nM). BAC cells were treated with or without $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern showing the two StAR transcripts, 3.0 and 1.8kb. The membrane was stripped and reprobed for β-actin. B: quantification of northern hybridisation shown is the sum of the two StAR transcripts and C: corrected for β-actin. Both graphs are mean ± SD from three independent cell isolations. Inserts show $ACTH_{1-24}$-treated levels normalised to untreated levels. *P<0.05 comparing untreated ands $ACTH_{1-24}$-treated levels.

5.2.5 Effect of $ACTH_{1-39}$ on StAR mRNA expression

The StAR transcript expression by BAC cells treated with 10nM $ACTH_{1-39}$ (figure 5.9) is comparable to that found with 10nM $ACTH_{1-24}$ (figure 5.7). The northern hybridisation displayed in the figure 5.9A shows a comparable pattern of expression of the two transcripts, 3.0 and 1.8kb, as found with 10nM $ACTH_{1-24}$. Quantification of the northern (figure 5.9B+C) showed an increase in the StAR mRNA levels at most of the time points studied. A peak in the StAR transcript levels was found at 4 hours, thereafter the levels of the StAR mRNA decreased to almost untreated levels by 12 hours of $ACTH_{1-39}$-treatment. This was reflected in the StAR levels when presented relative to the control values (inserts). At 6 hours $ACTH_{1-39}$-treatment BAC cells gave raise to a 6-fold increase compared with untreated cells. As this result was in agreement with the response found with $ACTH_{1-24}$-treated BAC cells and the cortisol response in these cells (figure 3.11), the experiment was performed only once.

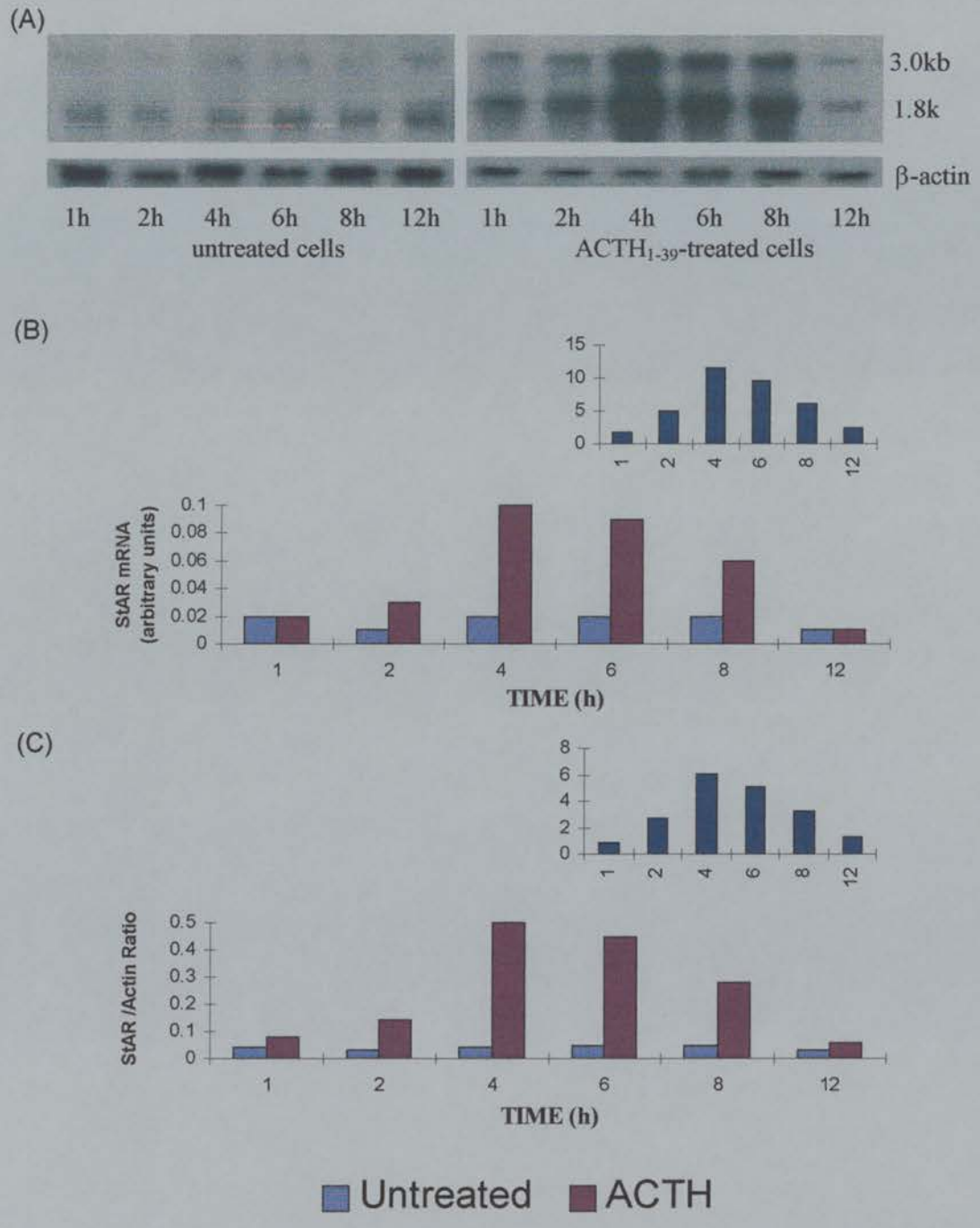


Figure 5.9. StAR mRNA expression in BAC cells treated with $ACTH_{1-39}$ (10nM). BAC cells were treated with or without $ACTH_{1-39}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern showing the two StAR transcripts, 3.0 and 1.8kb. The membrane was stripped and reprobed for β-actin. B: quantification of northern hybridisation shown is the sum of the two StAR transcripts and C: corrected for β-actin. Both graphs are from one cell isolation. Inserts show $ACTH_{1-39}$-treated levels normalised to untreated levels.

5.2.6 StAR mRNA expression in BAC cells cultured in DMEM/F12

BAC cells maintained in DMEM/F12 were shown to produce an equivalent cortisol response to 10nM $ACTH_{1-24}$-treatment to those maintained in Ham's F10, the medium of choice for the work carried out in this thesis (figure 4.3). To ascertain if the StAR mRNA expression was similar under both conditions BAC cells were maintained in DMEM/F12 as described in 4.2.3.

The results presented in figure 5.10A demonstrates that the StAR mRNA expression by BAC cells in response to 10nM $ACTH_{1-24}$- treatment was similar to that produced by cells maintained in Ham's F10. StAR mRNA (figure 5.7). Correspondingly, StAR transcripts were detected after 1 hour of $ACTH_{1-24}$- treatment, with a peak in expression around 6 hours, followed by a decrease in the levels by 12 hours. An increase in the levels was found at each of the time points when the northern was quantified by the BioRad phosphoimaging system. After 6 hours of $ACTH_{1-24}$-treatment a 3-fold increase in the StAR transcript levels was found compared with untreated cells (inserts). As this result was in agreement with the response found with $ACTH_{1-24}$-treated BAC cells and the cortisol response in these cells (figure 3.11), the experiment was performed only once.

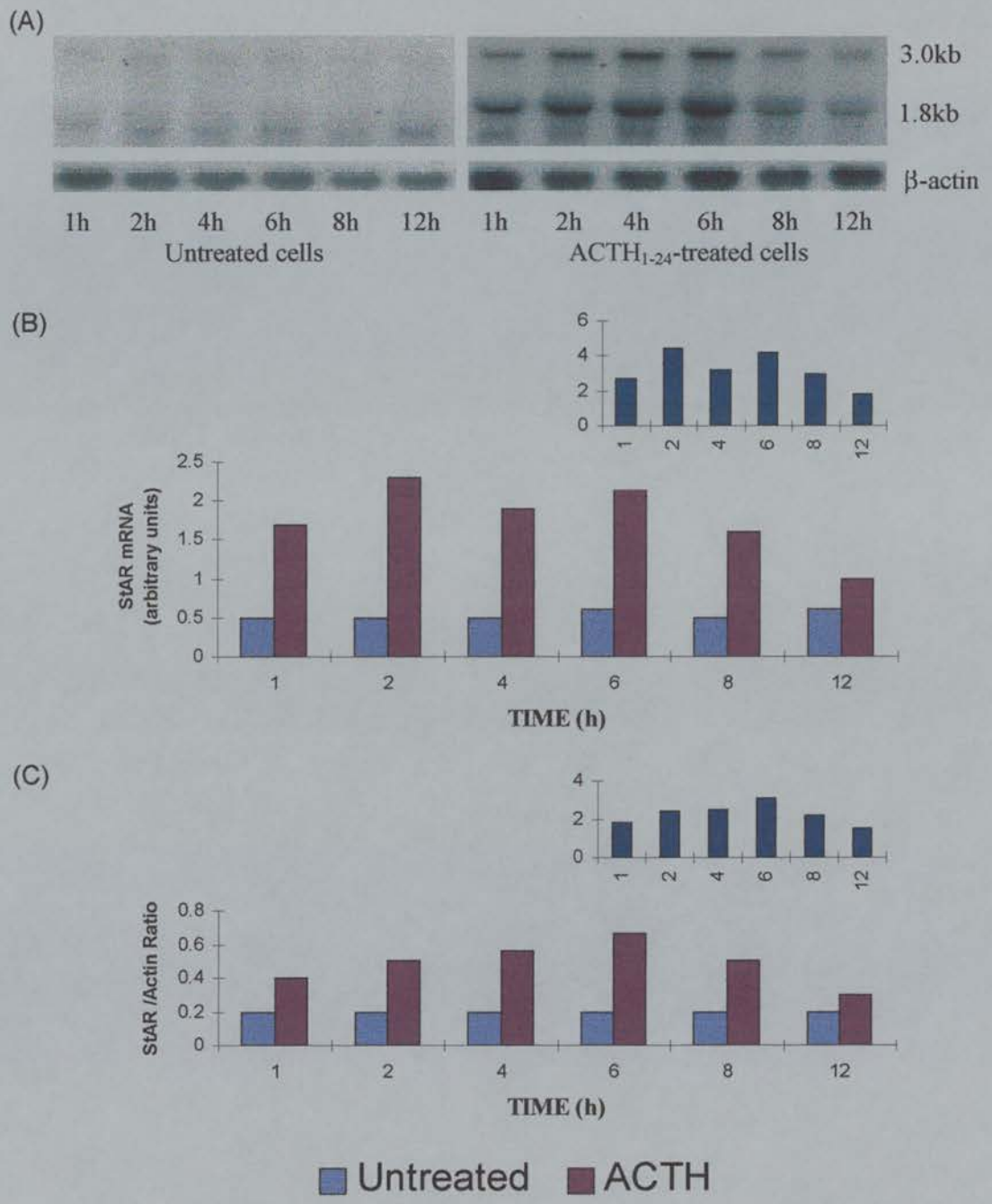


Figure 5.10. StAR mRNA expression in BAC cells maintained in DMEM/F12
BAC cells were treated with or without 10nM $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern showing the two StAR transcripts, 3.0 and 1.8kb. The membrane was stripped and reprobed for β-actin. B: quantification of northern hybridisation shown is the sum of the two StAR transcripts and C: corrected for β-actin. Both graphs are from one independent cell isolation. Inserts show $ACTH_{1-24}$-treated levels normalised to untreated levels.

5.2.7 StAR transcript expression in BAC cells treated with forskolin, 8Br-cAMP and AngII

The northern hybridisation of BAC cells treated with forskolin (10μM) showed that StAR mRNA was detected in the cells after 1 hour. There was a slight increase in the StAR transcript expression up to around 6 hours, followed by a slight decrease in expression through 12 hours (figure 5.11). The quantification of the northerns showed a significant increase in the amount of StAR expression in the forskolin-treated cells at 1, 2, 4, 6 and 8 hours compared with the untreated cells ($P<0.05$), there was no significant difference found at 12 hours. The peak in StAR transcript in forskolin-treated BAC cells occurred around 6 hours.

Figure 5.12 displays the expression of StAR mRNA in BAC cells treated with 8Br-cAMP (100μM). The northern hybridisation shows the presence of StAR mRNA in the cells after 1 hour of treatment. The expression of the StAR transcripts remained constant over the course of the experiment. When the data was quantified the levels of the StAR transcripts were found to be significantly increased above untreated levels at 4 hours only. When the data was normalised to the untreated cells (inserts) a constant level of StAR mRNA expression over the 12 hour time course was seen.

The StAR mRNA expression in BAC cells treated with AngII (10nM) is shown in figure 5.13. StAR mRNA is detected in the cells after 1 hour of AngII treatment. There was a significant increase in the StAR transcript levels in AngII-treated cells compared with untreated cells at 1, 2 and 4 hours ($P<0.05$). The peak in StAR transcript levels occurs at 4 hours of AngII treatment with a fall to untreated levels by 12 hours.

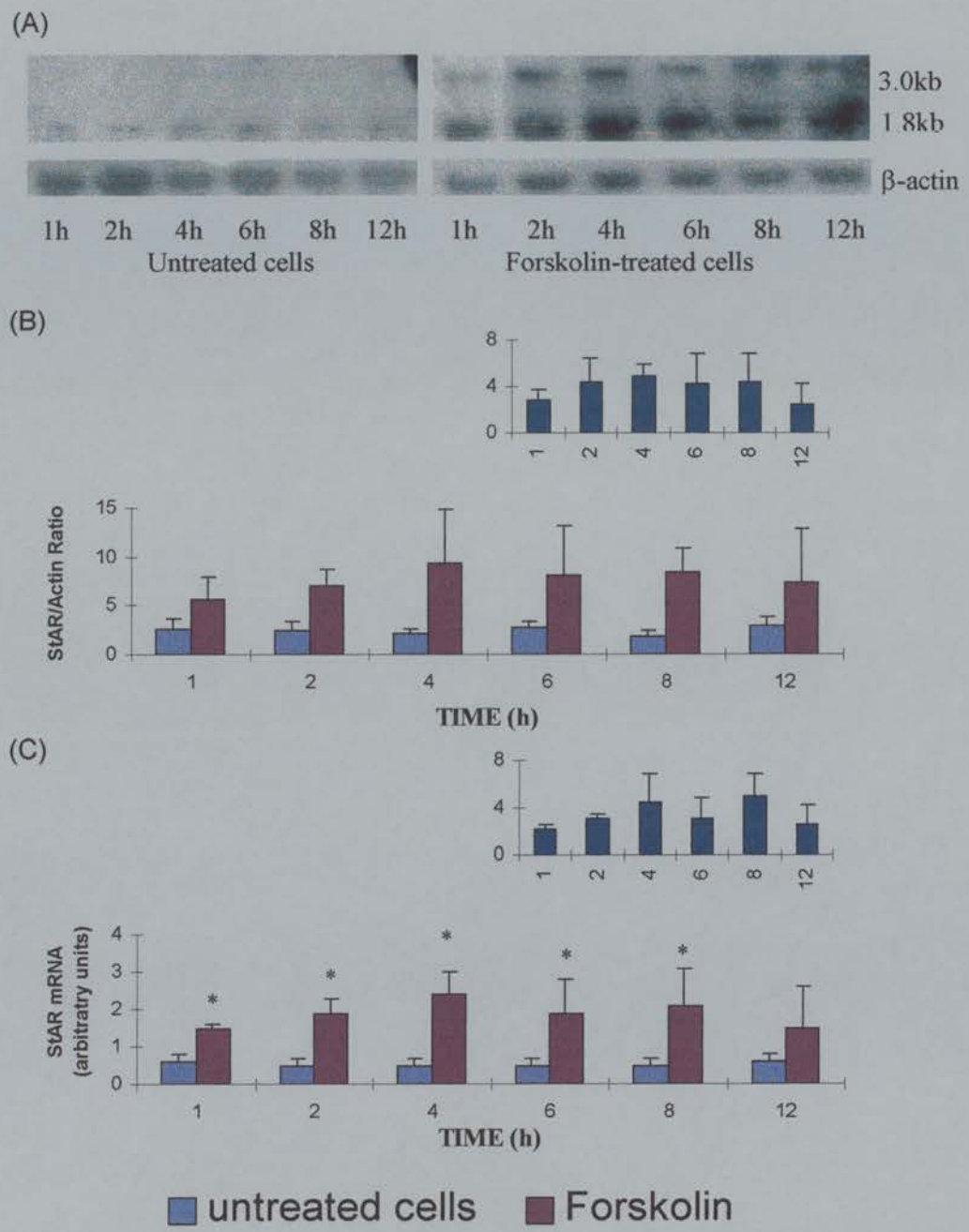


Figure 5.11. StAR mRNA expression in BAC cells treated with forskolin (10μM) The cells were treated with or without forskolin for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. 25μg total RNA/lane. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts C: corrected for β-actin. Results are mean ± SD from three independent cell isolations. Inserts show valuse normalised to untreated. *P<0.05 comparing untreated and forskolin-treated levels.

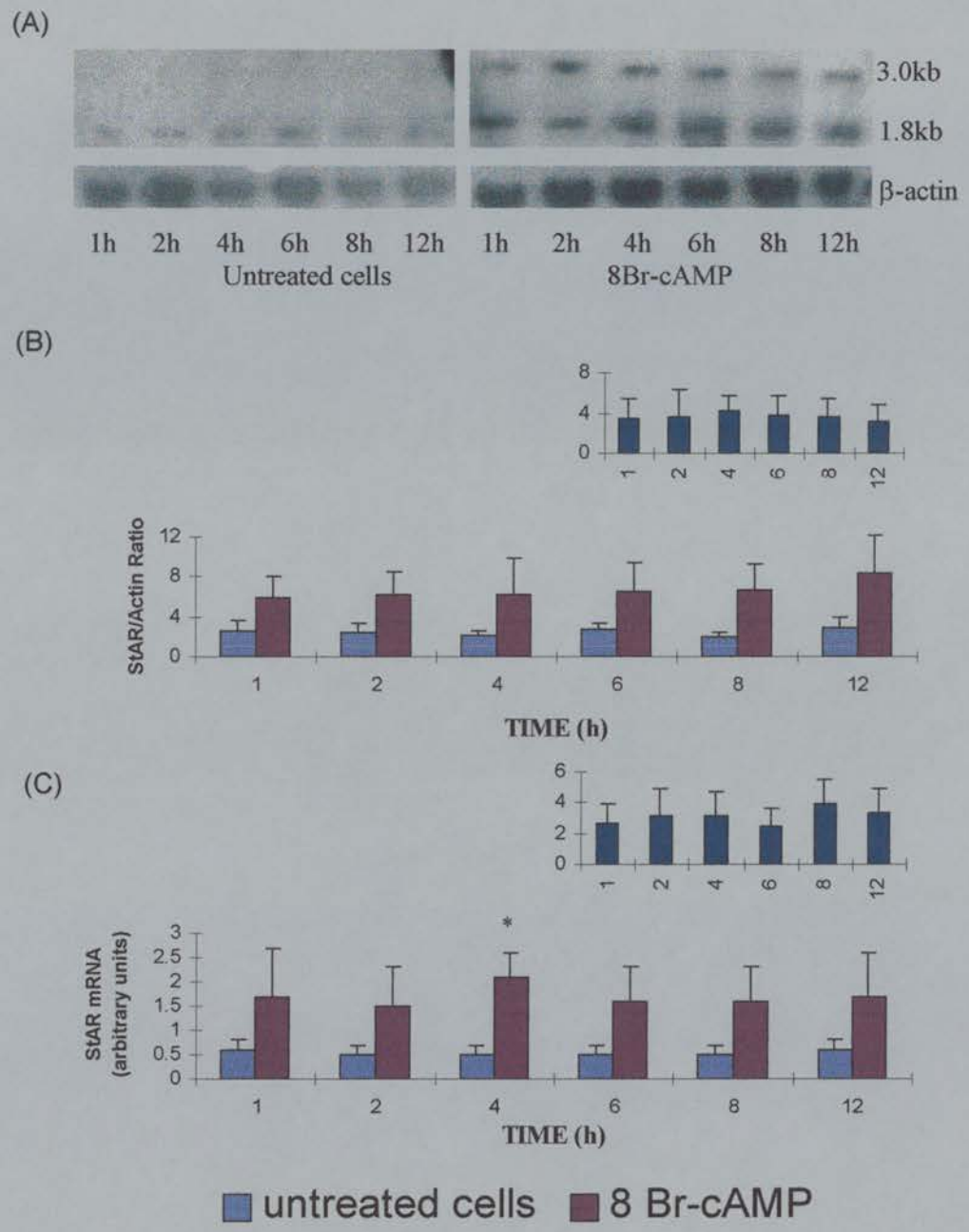


Figure 5.12. StAR mRNA expression in BAC cells treated with 8Br-cAMP (100μM). BAC cells were treated with or without 8Br-cAMP for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. 25μg total RNA/lane. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts and C: corrected for actin. Results are mean ± SD from three independent cell isolations. Inserts show values normalised to untreated. *P<0.05 comparing treated with untreated cells.

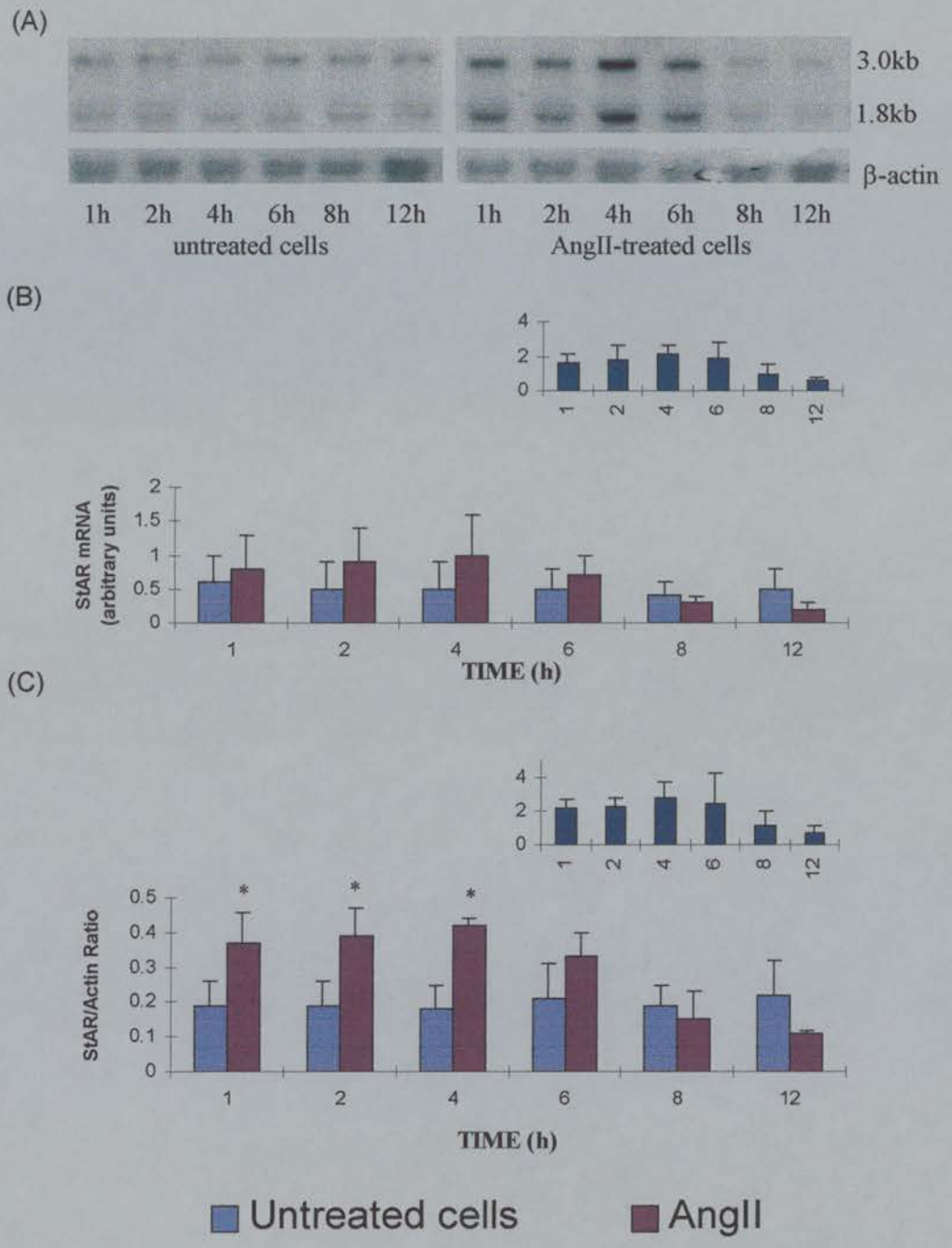


Figure 5.13. StAR mRNA expression in BAC cells treated with AngII (10nM). BAC cells were treated with or without AngII for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation. A, representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts and C: corrected for actin. Results are mean ± SD from three independent cell isolations. Inserts show values normalised to untreated. *P<0.05 comparing untreated and AngII-treated cells.

5.3 Discussion :

Northern hybridisation analysis of total BAC cells mRNA, using a [α^{32}P]-labeled cDNA probe encoding a 5'region of the bovine StAR mRNA (nucleotides 210-608) revealed two principal hydridising species, 3.0 and 1.8 kb. This is consistent with other studies using bovine corpora lutea (Pescador, 1996), (Hartung, 1995), bovine adrenal glomerulosa cells (Brand, 1998), (Cherradi, 1998b) and bovine fasiculata cells (Le Roy, 2000), (Ivell, 2000). The different transcript sizes found are due to alternative polyadenylation sites found in the cDNA sequence (Hartung, 1995).

The results presented in chapter 3 concluded that the optimum conditions for the study of cortisol release from BAC cells in response to ACTH-treatment was 48 hours after culture, with the cells serum-deprived overnight prior to experimentation. The results obtained in this chapter confirmed that these conditions were also optimum for the study of StAR mRNA expression in BAC cells.

StAR mRNA was expressed at high levels in both untreated and ACTH-treated cells when measured on day 0; however, the cortisol levels were found to be low. One explanation could be that the cells are in a state of stress due to the abattoir and the isolation procedure, with the levels of StAR mRNA increasing to initiate steroidogenesis to deal with these stresses. On the other hand the cells maybe incapable of producing cortisol due to the low number of lipid deposits present within the cells and damage to the cells' ultrastructure integrity caused by the isolation procedure (Williams, 1989). The StAR mRNA levels fall dramatically on day 1 of culture, although an increase in the expression of StAR mRNA is seen with 6 hours of ACTH-treatment. Compatible with the data obtained on the cortisol output the optimum expression of StAR mRNA was on day 3 of culture. The expression of StAR mRNA was found to decrease to almost untreated levels after 24 hours of ACTH-treatment on each of the days studied. A similar pattern of events was found with StAR protein, high levels were found in freshly isolated cells which

then decrease with time in culture. ACTH-treatment on days 1-4 in culture increased StAR protein levels at 6 hours, however after prolonged (24h) ACTH-treatment StAR protein levels returned to untreated levels (Nicol, 1998). In agreement with the cortisol data presented in chapter 3, there was no difference in the expression of the StAR transcripts by BAC cells treated with or without serum present in the cell culture medium. Similarly no difference was found in the StAR transcript expression when cells were cultured in DMEM/F12 compared with Ham's F10.

ACTH-treatment produced an increase in StAR mRNA expression with levels detected after 1 hour, reaching a maximum at around 4-6 hours and falling to near untreated levels by 12 hours treatment. A similar pattern of StAR mRNA expression was found with different concentrations of $ACTH_{1-24}$ and the native $ACTH_{1-39}$ peptide. From these results it appears that StAR mRNA levels in BAC cells are acutely regulated in concert with ACTH-stimulated cortisol secretion. This is consistent with studies in MA-10 Leydig tumor cells, where a marked increase in StAR mRNA was seen within 2 hours of dibutyryl-cAMP (dbcAMP)-treatment, with peak levels observed at 4-6 hours and levels falling to almost untreated levels by 12 hours (Clark, 1995b).

StAR protein has been shown to be regulated through the cAMP messenger pathway (Clark, 1995b). As mentioned above dbcAMP-treatment of MA-10 cells showed a similar pattern of StAR mRNA expression to that found in ACTH-treated BAC cells. The progesterone production in the MA-10 cells was also found to reach a maximum by 6 hours followed by decline in levels thereafter (Clark, 1995b). Treatment of BAC cells with forskolin and 8Br-cAMP showed no plateau in cortisol production (figure 3.12, 3.13), the effect of forskolin and 8Br-cAMP on StAR mRNA expression in BAC cells was therefore evaluated. StAR mRNA levels in BAC cells increased in response to forskolin-treatment. As seen in figure 5.9 StAR mRNA expression was detected at 1 hour, increasing to a maximum at around 6 hours, with the levels decreasing slightly thereafter. Detectable upregulation of StAR mRNA

was also seen within 1 hour of 8Br-cAMP treatment, thereafter the levels of StAR mRNA remained constant over the 12 hour period. 8Br-cAMP was shown to increase StAR protein levels significantly at 6 hours in BAC cells (Morley, 2000). The cAMP analogues, dbcAMP, has been shown to stimulate StAR protein in MA-10 cells (Huang, 1997) and in the human adrenocortical carcinoma cell line, H259 (Clark, 1995a) in a time dependent manner.

Studies performed in this thesis have also examined the effects of alternate second messenger pathways on StAR mRNA expression. The results presented in chapter 3 showed that AngII increased cortisol production in BAC cells; this is consistent with other studies (Clyne, 1993a), (Bird, 1989), (Walker, 1991b). AngII increased cortisol production over the initial 4 hours, with a plateau, similar to that seen with ACTH, occurring at around 4 hours. In BAC cells the expression of StAR mRNA was found to be upregulated within 1 hour of AngII-treatment, peaking at around 4 hours and declining to almost untreated levels by 12 hours. Studies using H295 cells also demonstrated an induction of StAR protein in response to AngII-treatment in a time-dependent manner (Clark, 1995a). The data presented in this chapter suggest that StAR expression is a common mechanism utilised by PKA and PKC second messenger systems for the acute regulation of steroid biosynthesis.

In summary, the rate-limiting step in steroidogenesis is the delivery of cholesterol into the mitochondria where it is converted to pregnenolone by CYP11A, which is dependent on the hormonally-induced StAR protein (Clark, 1994). ACTH-treatment of BAC cells gave rise to an increase in StAR mRNA expression, which reached a peak at around 4-6 hours followed by a decline in expression through12 hours. This pattern of expression parallels the response seen in cortisol production, which was found to increase over the initial 4-6 hours of ACTH-treatment after which the further synthesis in cortisol was not detectable. The results presented in sections 4.2.1 and 4.2.2 have shown that the BAC cells are still responsive to ACTH-treatment, therefore, the hypothesis of receptor desensitisation was ruled out. These

data also demonstrated that the lack of cholesterol was unlikely to be the cause of the decline in steroidogenesis. The question as to whether this second steroidogenic response is also mediated via the transcription of StAR requires further investigation.

These data raise the question as to whether the decline in steroidogenesis is a result of the decline in StAR transcription and if so, what causes this decline in StAR transcription. One possibility is the presence of a local negative feedback inhibition mechanism with in the BAC cell culture system. A study in by Morrow et al in 1967 suggested that steroids were capable of inhibiting adrenal steroid production by inhibition of adrenal protein synthesis (Morrow, 1967). Therefore the possibility that cortisol, or one of the other steroids produced by BAC cells, has an inhibitory effect on StAR transcription was intriguing.

CHAPTER 6 : FURTHER CHARACTERISATION OF StAR mRNA EXPRESSION IN BAC CELLS.

6.1 Introduction

Cortisol is produced by the adrenal cortex under the regulatory influence of ACTH. The latter is produced by corticotrophins of the anterior pituitary, in turn, under the regulatory influence of hypothalamic CRH (see chapter 1.2). The hypothalamic-pituitary-adrenal (HPA) axis is kept in balance by the negative feedback effects of cortisol on the secretion of ACTH and CRH. ACTH was one of the first hormones found to stimulate steroidogenesis through a specific cell surface receptor via the cAMP second messenger pathway (Lefkowitz, 1970). In adrenal cells, the ACTH receptor exhibits a highly specific affinity for ACTH and ^{125}I-ACTH was not displaced by other melanocortins such as αMSH or βMSH. Contrary to most of the peptide hormone receptors, which are desensitised by their own ligand, the ACTH receptor is positively regulated by ACTH (Penhoat, 1994). Moreover, studies have shown that ACTH can regulate AngII receptors in the zona glomerulosa (Andoka, 1984), and that AngII can influence the ACTH receptor in zona fasiculata/reticularis cells (Le Roy, 2000), (Penhoat, 1995).

Most known effects of glucocorticoids are mediated by a ~94kDa intracellular protein, the GR (see chapter 1 section 1.4.5). Some studies have indicated that glucocorticoids may directly affect the function of the adrenal gland. A direct inhibitory effect of glucocorticoids on its own synthesis at the adrenal level has been suggested by several researchers (Peron, 1960), (Salmenperae, 1976). An inhibitory effect of corticosterone on steroidogenesis in the mouse adrenal tumor cell line Y-1 has also been demonstrated. Steroids which do not possess glucocorticoid action did not inhibit steroidogenesis. (Saito, 1979). The binding of Synacthen ($ACTH_{1-24}$) to partially purified bovine adrenocortical plasma membrane receptors was found to be

inhibited by cortisol, but not its immediate precursor 11-deoxycortisol. Suggesting that cortisol may regulate its own synthesis by inhibiting binding of ACTH to adrenocortical cells (Latner, 1977).

A number of drugs can be used in the suppression of ACTH-induced steroidogenesis by interfering with one or more of the enzymatic steps. These compounds are useful in the characterisation of the processes involved in steroidogenesis and in therapy of glucocorticoid excess. Ketoconazole and etomidate are members of a class of imidazole-derived antifungal drugs, which in the early 1980s were found to have antisteroidogenic actions in humans (Orth, 1992). The effects of these compounds were first noticed in human testosterone production because the men taking the drug developed gynaecomastia. Ketoconazole acts in the adrenal by primarily inhibiting CYP11B1, it can also inhibit CYP11A (Orth, 1992) and CYP17 (Englelhardt, 1991). Etomidate also inhibits CYP11B1, but at higher doses can also inhibit CPY11A. Trilostane is an inhibitor of 3β-HSD, but has no effect on 11β, 17α or 21-hydroxylation, and is used in the treatment of breast cancer (Orth, 1992).

The regulation of StAR expression by trophic hormones and other steroidogenic agents has been extensively studied. In virtually every system studied, agents that caused an increase in steroid biosynthesis also increase the expression of the StAR protein. Thus steroid production and StAR expression has been found to be up-regulated by ACTH (Nishikawa, 1996), (Nicol, 1998); CRH (Huang, 1997); cAMP analogs (Clark, 1995a), (Huang, 1997), (Balasubramanian, 1997) and AngII (Clark, 1995a). The StAR gene is also subject to negative regulation and studies have shown that compounds such as diethylumbelliferyl (Choi, 1995), transforming growth factor-β_1 (Brand, 1998), atrial natriuretic peptide (Cherradi, 1998b), tumor necrosis factor α (Mauduit, 1998); interleukin 1β (Ogilvie, 1999); econazole (Walsh, 2000a) and various pesticides (Walsh, 2000d), (Walsh, 2000b), [Walsh, 2000c) cause disruption of the StAR gene.

The results presented in section 4.2.1 of this thesis have demonstrated that the ACTH receptor in BAC cells did not become desensitised after 24 hours of ACTH-treatment. The response by BAC cells to a second ACTH challenge was equivalent to that seen in cells without 24 hour pre-treatment with ACTH. One of the aims of this chapter was to investigate the effect of 24 hour ACTH pre-treatment on the StAR transcript expression when the cells are re-challenged with ACTH. In addition, the effect of AngII on the steroid output and StAR transcript expression was investigated.

As described above ACTH has been shown to have a positive effect on its own receptor, and was therefore ruled out as an explanation for the plateau found in cortisol secretion by BAC cells. Other studies have also shown a direct inhibitory effect of glucocorticoids on its own synthesis at the adrenal level. Therefore, the effect of cortisol and cortisone, in the culture medium of BAC cells, on steroid secretion and StAR transcript expression was also investigated in this chapter.

Upregulation of StAR mRNA is implicated in the rapid synthesis and secretion of steroids by steroidogenic cells in response to trophic hormones. Finally, in this chapter, inhibitors were used to prevent cortisol production by inhibiting different stages of the steroidogenic pathway. The effect of this inhibition on the StAR transcript expression produced by ACTH-treated BAC cells was investigated.

6.2 Results

6.2.1 StAR mRNA expression in BAC cells in response to repeat $ACTH_{1-24}$-treatment.

BAC cells were treated with or without $ACTH_{1-24}$ (10nM) for 24 hours, the medium was removed and the cells were washed with EBSS. The cells, regardless of 24 hour pre-treatment, were then treated with $ACTH_{1-24}$ (10nM) and samples were taken at various points over a 12 hour time course.

Figure 6.1 shows the results of two independent cell isolations. The representative northern hybridisation shows the two StAR transcripts at 3.0 and 1.8kb. The northern shows that the StAR mRNA expression is similar under both conditions. With the StAR transcripts being detected after 1 hour under both conditions. An increase in the transcripts was seen in both conditions, peaking at around 4-6 hours followed by a decline thereafter. The membrane was stripped and reprobed for the β-actin gene to ensure there was equal loading and transfer of the RNA samples.

Quantification of the two experiments is displayed in figure 6.1B&C. BAC cells maintained as normal and treated with $ACTH_{1-24}$ show the familiar pattern, with a peak in StAR transcript levels found at 6 hours. The response found in BAC cells treated with $ACTH_{1-24}$ for 24 hours prior to the 12 hour $ACTH_{1-24}$-treatment paralleled that found under the normal 12 hour $ACTH_{1-24}$-treatment. After 1 hour of $ACTH_{1-24}$-treatment the StAR transcripts were detectable, with a peak in transcript expression found between 4 and 6 hours of $ACTH_{1-24}$-treatment, with a decline in levels thereafter. This was in keeping with the results obtained for the cortisol, where no difference was found between the two conditions (figure 4.1).

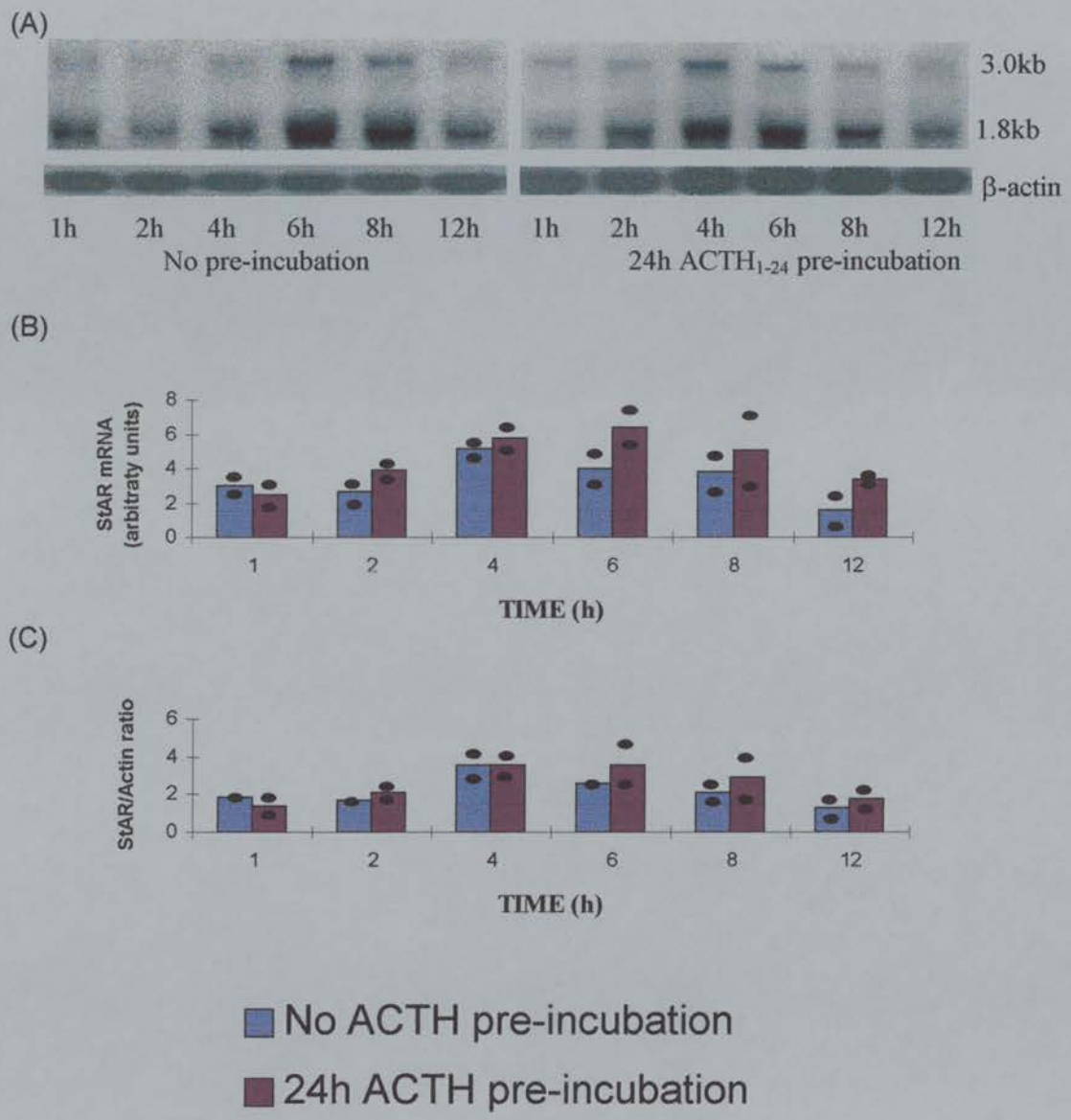


Figure 6.1. StAR mRNA expression in BAC cells re-treated with $ACTH_{1-24}$. BAC cells were treated with or without $ACTH_{1-24}$ for 24 hours then recharged with $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A, Representative northern analysis showing the 3.0 and 1.8 kb bands. B, Quantification of northern hybridisations, results are mean from two independent cell isolations (dots indicating value for each experiment). Upper panel is the sum of the two transcripts, the lower panel is corrected for β-actin.

6.2.2 Effect of repeated 12 hour $ACTH_{1-24}$ treatments on StAR transcript expression.

BAC cells were treated with or without $ACTH_{1-24}$ for 72 hours, the medium was changed every 12 hours. Samples for untreated cells were taken at 24, 48 and 72 hours only.

BAC cells harvested after 12 hours of $ACTH_{1-24}$-treatment show detectable StAR mRNA levels (figure 6.2) upon northern hybridisation. The expression of the StAR transcripts appeared to fall at 24 hours of $ACTH_{1-24}$-treatment and recover slightly thereafter.

When the StAR transcripts were quantified, the levels of StAR mRNA expressed in the cells over this time period were found not to be significantly different from the untreated levels at 24, 48 and 72 hours. There was no significant difference in the expression of the $ACTH_{1-24}$-treated StAR transcripts over the course of the experiment.

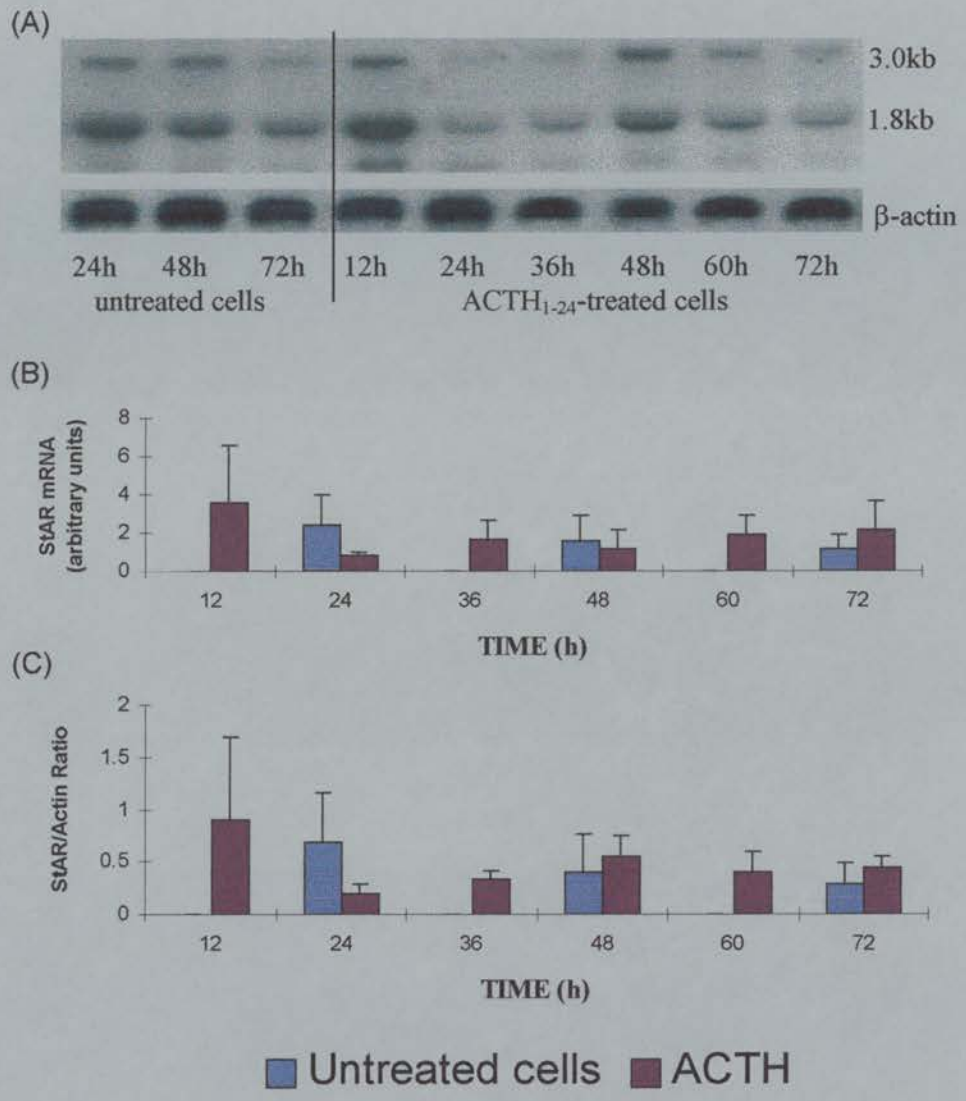


Figure 6.2. StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ every 12 hours. BAC cells were treated with or without $ACTH_{1-24}$ for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts and C: corrected for β-actin. Results are mean ± SD from three independent cell isolations.

6.2.3 The effect of AngII on the $ACTH_{1-24}$-induced steroid response.

The cortisol output by BAC cells in response to treatment with AngII with or without $ACTH_{1-24}$ present is displayed in figure 6.3. BAC cells treated with AngII show a significant increase in the cortisol secreted into the overlying medium compared with untreated cells at each of the time points studied ($P<0.05$). A similar increase in cortisol secretion over untreated was also found in the $ACTH_{1-24}$-treated cells ($P<0.01$ at all time points), and in cells treated with both AngII and $ACTH_{1-24}$ ($P<0.01$). Fitting a nonlinear curve equation to determine when the cortisol output reaches a plateau, it was found that for all three conditions the plateau was reached at around 4 hours of treatment.

$ACTH_{1-24}$ was found to secrete more cortisol into the medium overlying the cells than AngII at all of the time points studied ($P<0.05$). Thus indicating that ACTH was a more potent stimulus in BAC cells compared with AngII. Similarly $ACTH_{1-24}$/AngII produced significantly more cortisol than AngII alone ($P<0.05$). However, there was no significant difference found in the amount of cortisol secreted by BAC cells treated with ACTH compared with $ACTH_{1-24}$/AngII treatment.

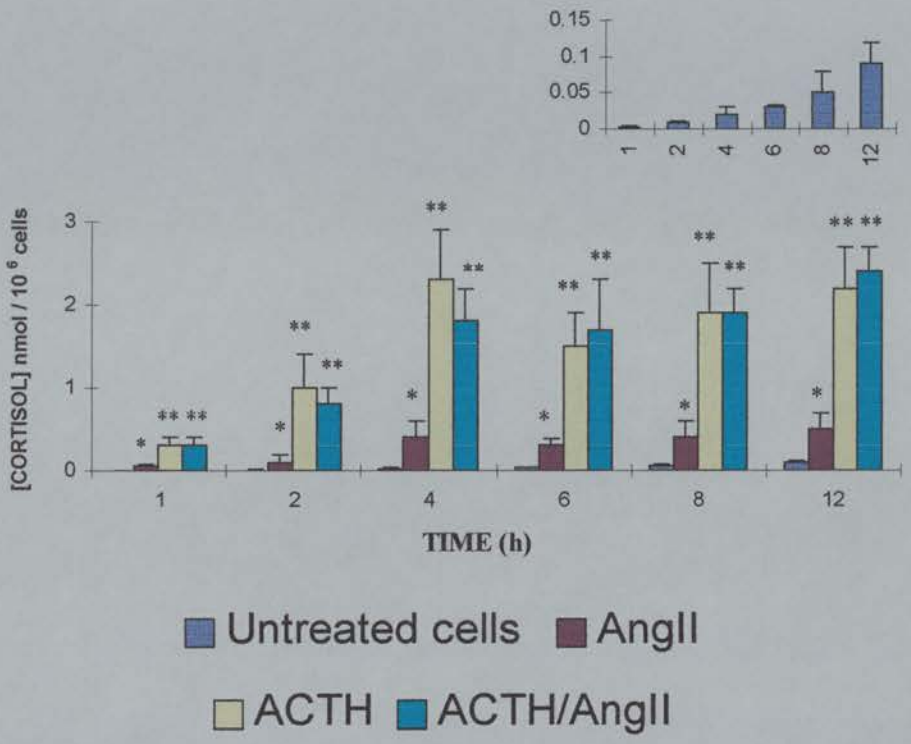


Figure 6.3. Effect of AngII on the cortisol output by $ACTH_{1-24}$-treated BAC cells.

BAC cells were treated as indicated, the medium was removed and assayed for cortisol. Results shown are mean ± SD from three independent cell isolations. Insert shows untreated levels. *P<0.05, **P<0.01 comparing untreated with treated cells at each time point.

6.2.4 The effect of AngII on the expression of the StAR transcripts in $ACTH_{1-24}$-treated BAC cells.

Figure 6.4A shows a representative northern analysis of BAC cells treated with AngII, $ACTH_{1-24}$ and AngII/ $ACTH_{1-24}$. The response by BAC cells to AngII- and $ACTH_{1-24}$-treatement was found to be similar to that already present within this thesis. With $ACTH_{1-24}$ there was a significant increase in the expression of the StAR transcripts within 2 hours ($P<0.05$), with a peak in expression found between 4 and 6 hours (16-fold compared with untreated at 6 hours). The levels falling to almost untreated levels by 12 hours (2-fold compared to untreated). AngII-treatment of BAC cells displayed a significant increase in the StAR transcript levels compared with untreated cells at 1, 2 and 4 hours ($P<0.05$). The peak in the expression of the StAR transcripts by AngII-treated cells occurred around 4 hours (3-fold increase compared to untreated), with a decline to untreated levels by 12 hours.

The results provided by the representative northern hybridisation suggest that when $ACTH_{1-24}$ and AngII were used together to treat BAC cells the increase in StAR transcript expression is less than that found with $ACTH_{1-24}$ alone. The quantification data showed an increase in the expression of the StAR transcripts compared with untreated cells, a 7-fold increase compared with untreated at 6 hours, although this was found to be significant at 2 hours of treatment only ($P<0.05$). The expression of the StAR transcript was found to be lower in the cells treated with both agonists than with $ACTH_{1-24}$ alone. However, this was not found to be significant (figure 6.5).

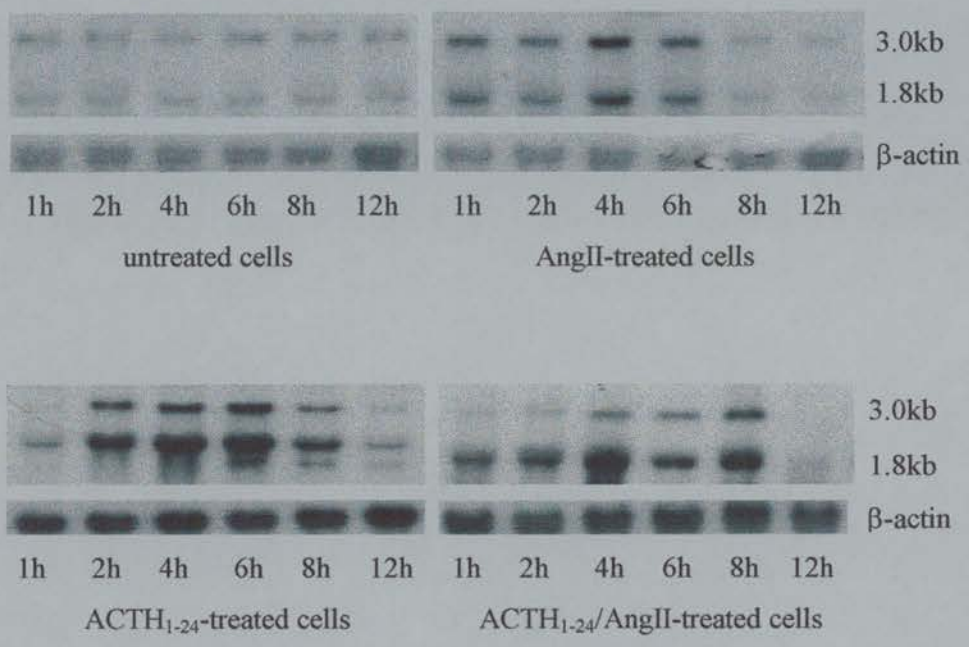


Figure 6.4 Representative northern analysis of StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ and/or AngII. BAC cells were treated as indicated above. RNA was extracted from the cells and using 25μg total RNA/lane subjected to northern hybridisation using a bovine StAR cDNA probe. Two transcripts were found; 3.0 and 1.8 kb. The membrane was stripped and reprobed for β-actin

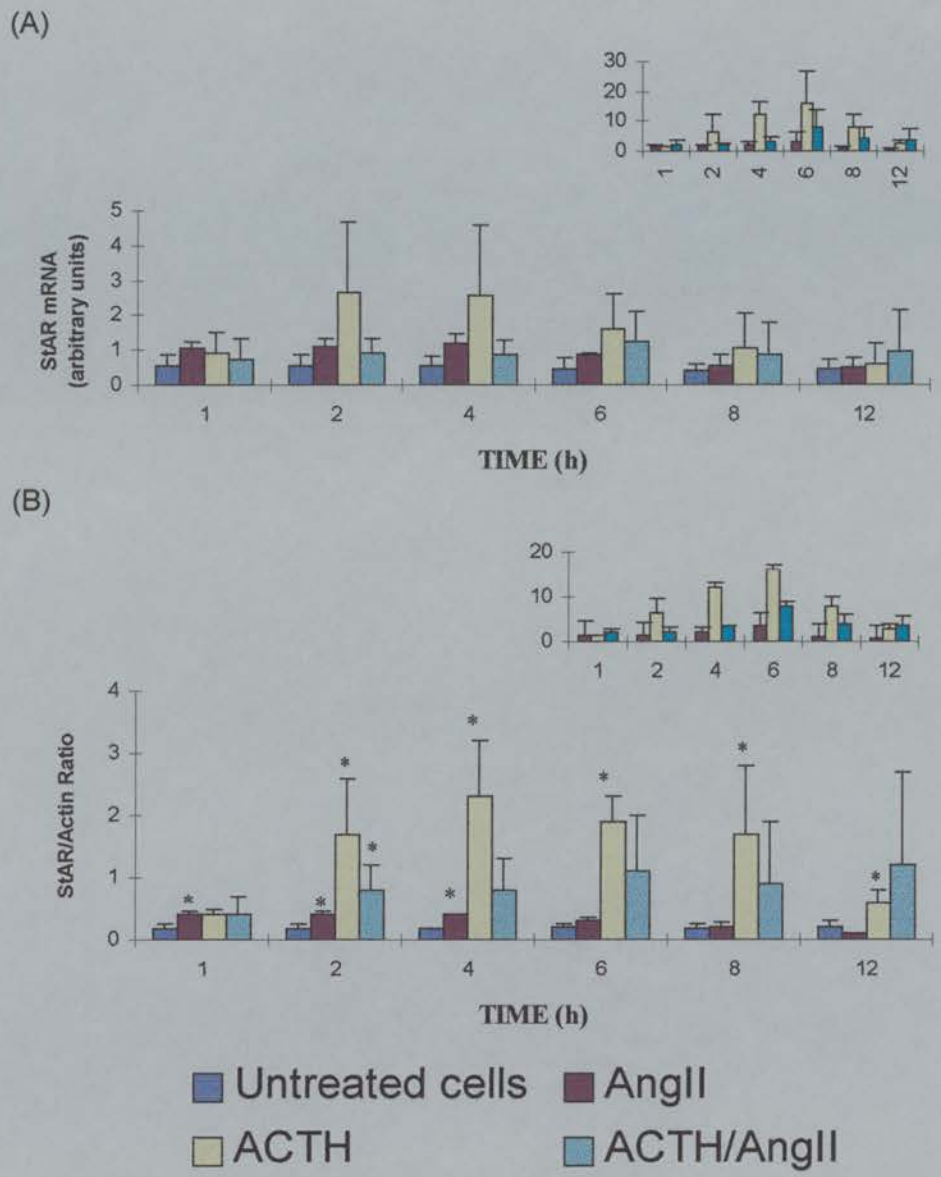


Figure 6.5 Quantification of northern hybridisation. StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ and/or AngII. A, Sum of the two StAR transcripts shown in figure 6.10. B, The sum of the two StAR transcripts corrected for actin. Mean ± SD from three independent cell isolations. Inserts shown values normalised to untreated cells.

6.2.5 Effect of cortisol on the steroid output in cultured BAC cells

To investigate if cortisol was having any effect on the cortisol output a level of cortisol corresponding to the maximum amount secreted by the cells at 6 hours was added to the cell culture medium. BAC cells were treated with or without $ACTH_{1-24,}$ (10nM) in the presence or absence of 5μM cortisol.

For the cells treated without added cortisol the picture was as previously reported (figure 6.6A). There was a significant increase in the production of cortisol when cells are treated with $ACTH_{1-24}$ compared to untreated cells at all time points studied ($P<0.01$). The plateau in cortisol output occurred after 6 hours of $ACTH_{1-24}$-treatment.

With the addition of cortisol to the medium, the level of cortisol at the start of the experiment (0h) was 5.5 (± 0.5) nmol/10^6 cells. This appeared to decrease over the course of the experiment, with a significant fall in the amount of cortisol in the overlying medium seen at 8 and 12 hours compared with the 1 hour levels ($P<0.05$). A significant difference in the levels of cortisol between the untreated cells and $ACTH_{1-24}$-treated cells was found at the later time points, ($P<0.05$).

BAC cells treated with 10nM $ACTH_{1-24}$ in the presence of 5μM cortisol displayed an increase in cortisol output over and above that found in the medium at 0 hour. Although this was found to be significant only at 4 and 8 hours.

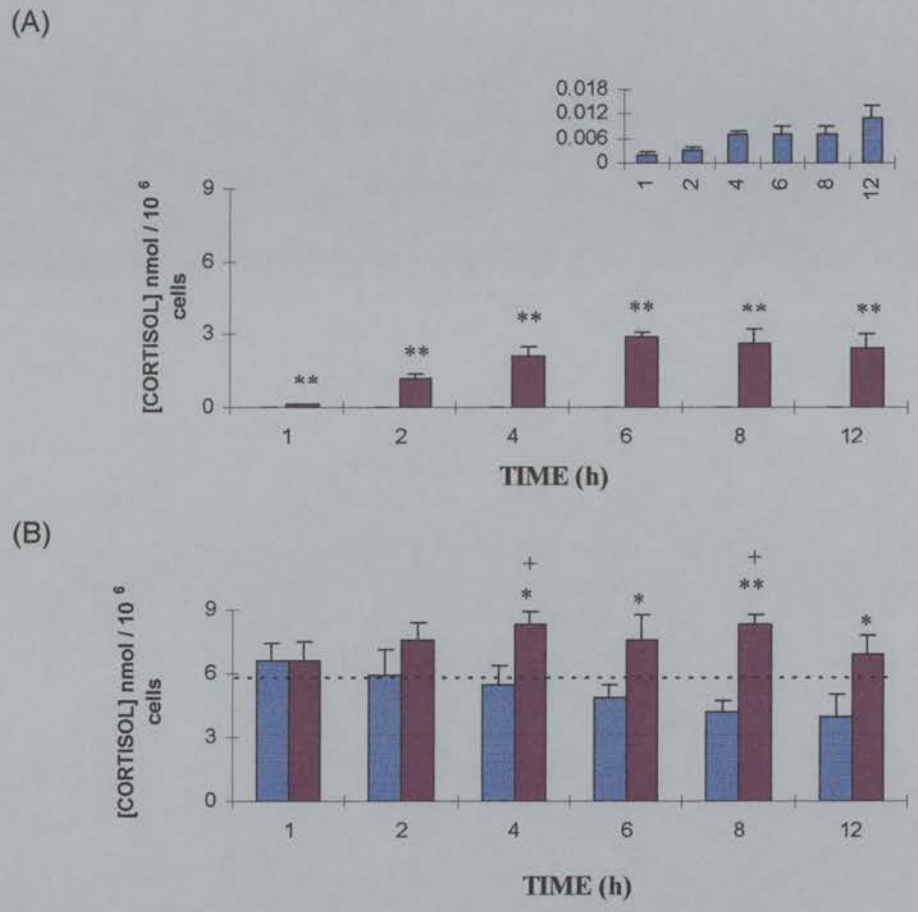


Figure 6.6. Cortisol added to the cell culture medium. BAC cells are treated with or without $ACTH_{1-24}$(10nM) in the A, absence or B, presence of 5μM cortisol. Results shown are three independent cell isolations. *P<0.05, **P<0.01 comparing untreated cells with $ACTH_{1-24}$-treated cells. Insert shows the untreated values in more detail. +P<0.05 comparing ACTH-treated cells with 0 hour time point. The broken line indicates the level of cortisol in the medium at the start (0h) of the experiment.

Cortisone and corticosterone were found upon HPLC analysis of the medium overlying the cells (figure 6.7). Cortisone was seen after 6 hours of $ACTH_{1-24}$-treatment and increased slightly to 12 hours. Corticosterone was detected after 1 hour of $ACTH_{1-24}$-treatment. There was significant increase in the amount of corticosterone found in the $ACTH_{1-24}$-treated cells over the ensuing hours compared with the value found at 1 hour ($P<0.05$). However, there was a plateau in the corticosterone output at 4 hours of $ACTH_{1-24}$-treatment.
11β-hydroxyandrostenedione was not detected at any of the time points studied.

Cortisol was detected in the untreated cells, due to the presence of 5μM cortisol in the medium, this was found to decrease with time as observed with the RIA data. Cortisone was also detected in the untreated cells after 4 hours, whereas previously no steroids were found in the untreated cells (chapter 4). The appearance of cortisone in the medium overlying the untreated cells corresponds to the decrease in the cortisol levels. The level of cortisone detected in the overlying medium after 12 hours $ACTH_{1-24}$-treatment was found to be half the value obtained for cortisol, 1.2 ±0.1 and 2.4 ±0.5nmol/10^6 cells respectively.

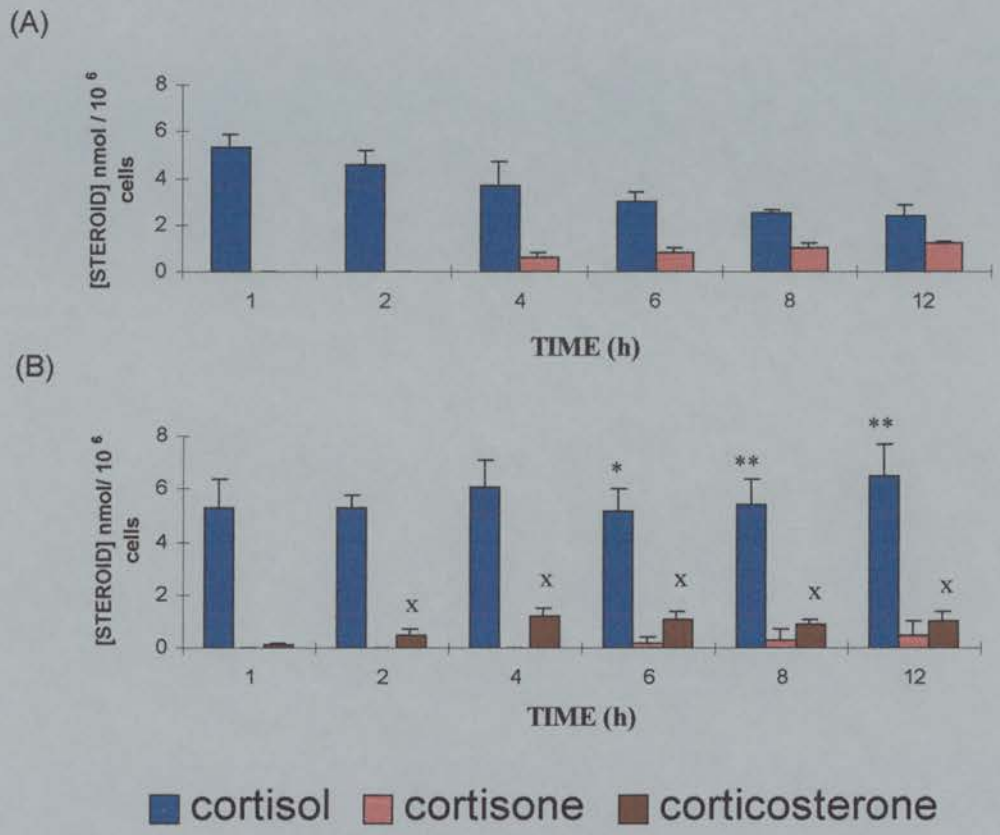


Figure 6.7 HPLC analysis of medium with cortisol added. Cells are treated A: without or B: with $ACTH_{1-24}$ (10nM) in the presence of 5μM cortisol. Results shown are mean ±SD of three independent cell isolations. *P<0.05, **P<0.01 comparing untreated with $ACTH_{1-24}$-treated cells at each time point. ^{x}P<0.05 comparing 1 hour corticosterone values with other time points.

6.2.6 Effect of cortisone on the steroid output in cultured BAC cells

The presence of cortisone in the culture medium did not have any effect on the cortisol secretion when the cells are treated with or without $ACTH_{1-24}$ (figure 6.8). A level of 5μM was chosen in keeping with cortisol in the previous experiment. Figure 6.8A illustrates the cortisol response by BAC cells to $ACTH_{1-24}$-treatment under normal conditions (i.e. no added steroids). A significant increase in the levels of cortisol produced by $ACTH_{1-24}$-treated cells was found compared with untreated cells in the presence of cortisone ($P<0.05$). The levels of cortisol secreted into the overlying medium of $ACTH_{1-24}$-treated cells, in the presence of cortisone was found to be similar to that observed under normal circumstances.

A slight increase in the amount of cortisol secreted into the overlying medium was noted at the later time points, 3.7 ±0.5 and 3.1 ±0.9 at 8 and 12 hours respectively in the presence of cortisone compared with 2.6 ±0.6 and 2.4 ±0.6 at 8 and 12 hours respectively in the absence of cortisone; however, no significant difference between the two conditions was seen at any of the time point studied. the plateau in cortisol output was found to occur around 6 hours with both conditions.

Analysis of the medium overlying the cells by HPLC revealed the presence of corticosterone and 11β-hydroxyandrostenedione in addition to cortisol and cortisone in $ACTH_{1-24}$-treated cells (figure 6.9), this is in keeping with the results presented in figure 3.7. $ACTH_{1-24}$-treated BAC cells produced cortisol and corticosterone in the presence of cortisone, over the initial 4 hours of ACTH-treatment in equal amounts. 11β-hydroxyandrostenedione was not detected in the cells until 4 hours and remain constant through 12 hours. From the data presented in figure 6.9A, the cortisone levels in untreated cells appear to decreased over the 12 hour period.

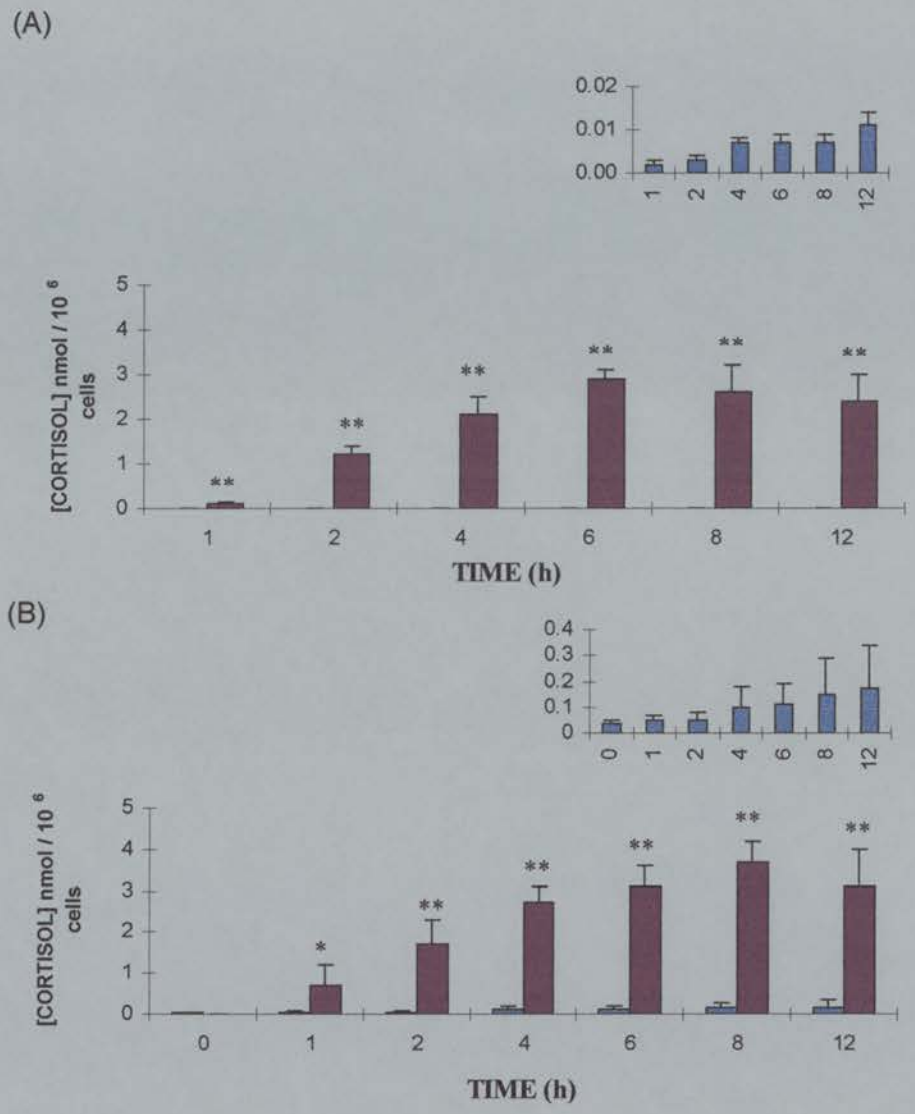


Figure 6.8. Cortisone added to the cell culture medium. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) in the A, absence or B, presence of 5μM cortisone. Insert shows basal values in more detail. Results shown are mean ±SD from three independent cell isolations. *P<0.05, **P<0.01, comparing untreated with $ACTH_{1-24}$-treated cells.

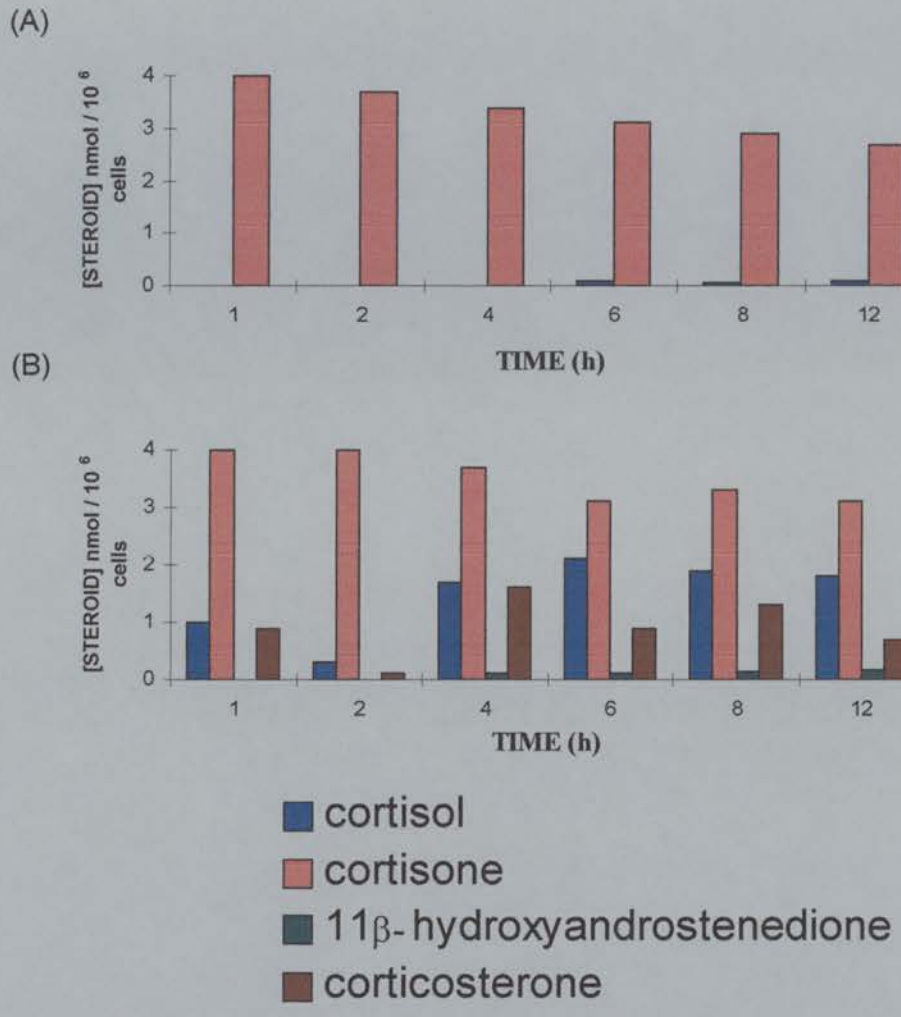


Figure 6.9. HPLC analysis of cortisone added to the cell culture medium. BAC cells were treated A, without or B, with $ACTH_{1-24}$ (10nM) in the presence of 5μM cortisone. Results shown are from one cell isolations.

6.2.7 Effect of cortisol and cortisone on StAR transcript expression in cultured BAC cells

The presence of 5μM cortisol had no effect on the expression of the StAR transcripts on 10nM $ACTH_{1-24}$-treated cells. The representative northern hybridisation (figure 6.10A) displays a similar pattern of expression to that previously described for 10nM $ACTH_{1-24}$-treated cells (figure 5.7). With detectable levels of the StAR transcripts found at 1 hour $ACTH_{1-24}$-treatment. The levels of the transcripts appear to peak at 4 hours with a decrease to almost untreated levels by 12 hours. Quantification of the StAR mRNA levels demonstrated an increase over untreated levels at 2 hours (3-fold compared with untreated cells), with a peak at around 6 hours (4-fold increase) and decreasing again by 12 hours of $ACTH_{1-24}$-treatment, however none of these were found to be significantly different from the untreated levels (figure 6.10).

Figure 6.11A displays a representative northern hybridisation of the StAR transcript expression in BAC cells treated with 10nM $ACTH_{1-24}$ in the presence of cortisone. The levels of the StAR transcripts were detected after 1 hour of $ACTH_{1-24}$-treatment and a significant increase ($P<0.05$) in the transcript levels, compared with untreated cells, was found at time points thereafter. A peak in expression occurred around 6 hours (4-fold compared with untreated cells), with a decline in the levels of the StAR transcripts thereafter, but remaining significantly increased over the untreated levels at 12 hours ($P<0.05$).

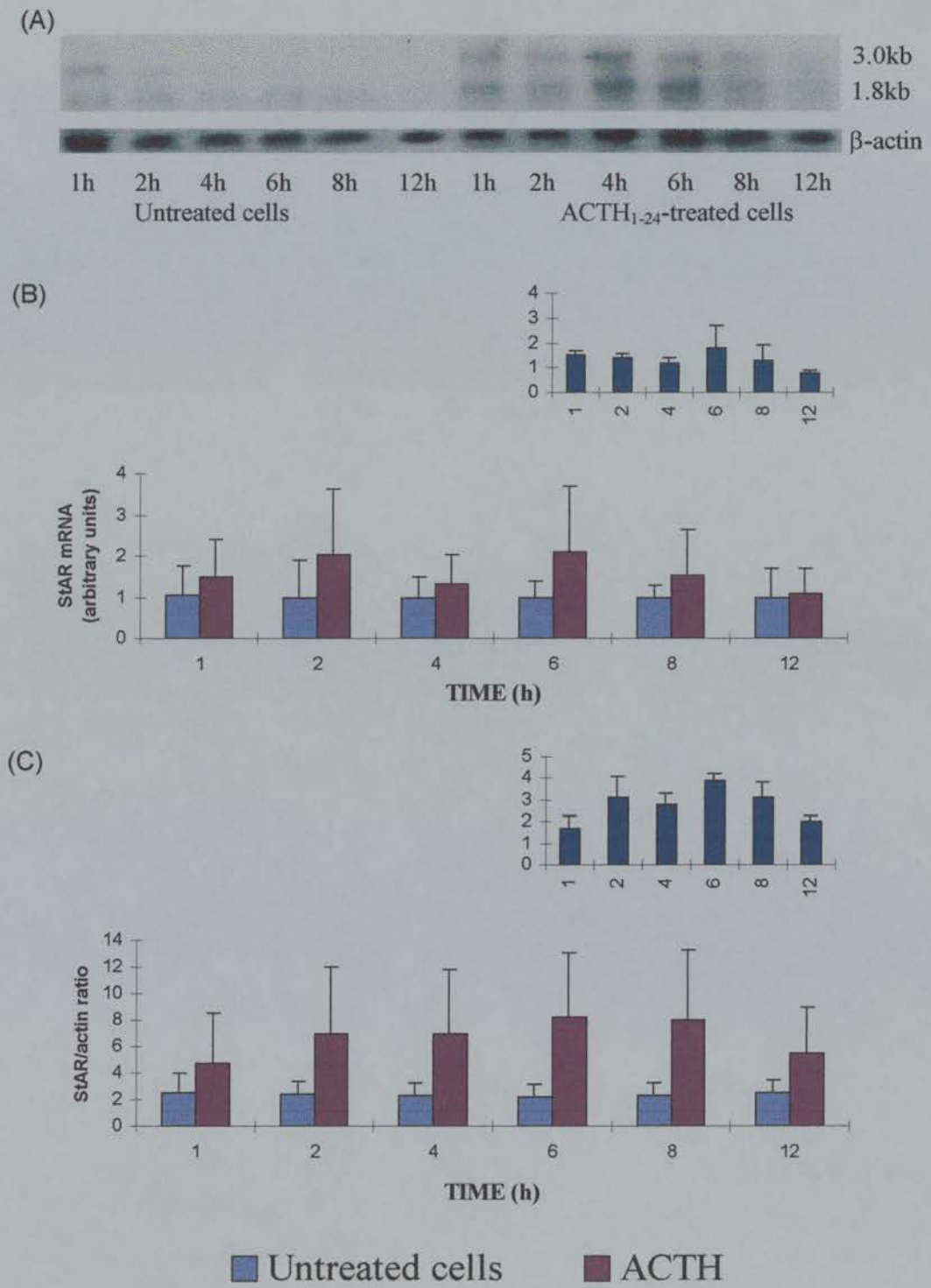


Figure 6.10 StAR mRNA expression in the presence of 5μM cortisol. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts and C: corrected for β-actin. Results are mean ± SD from three independent cell isolations.

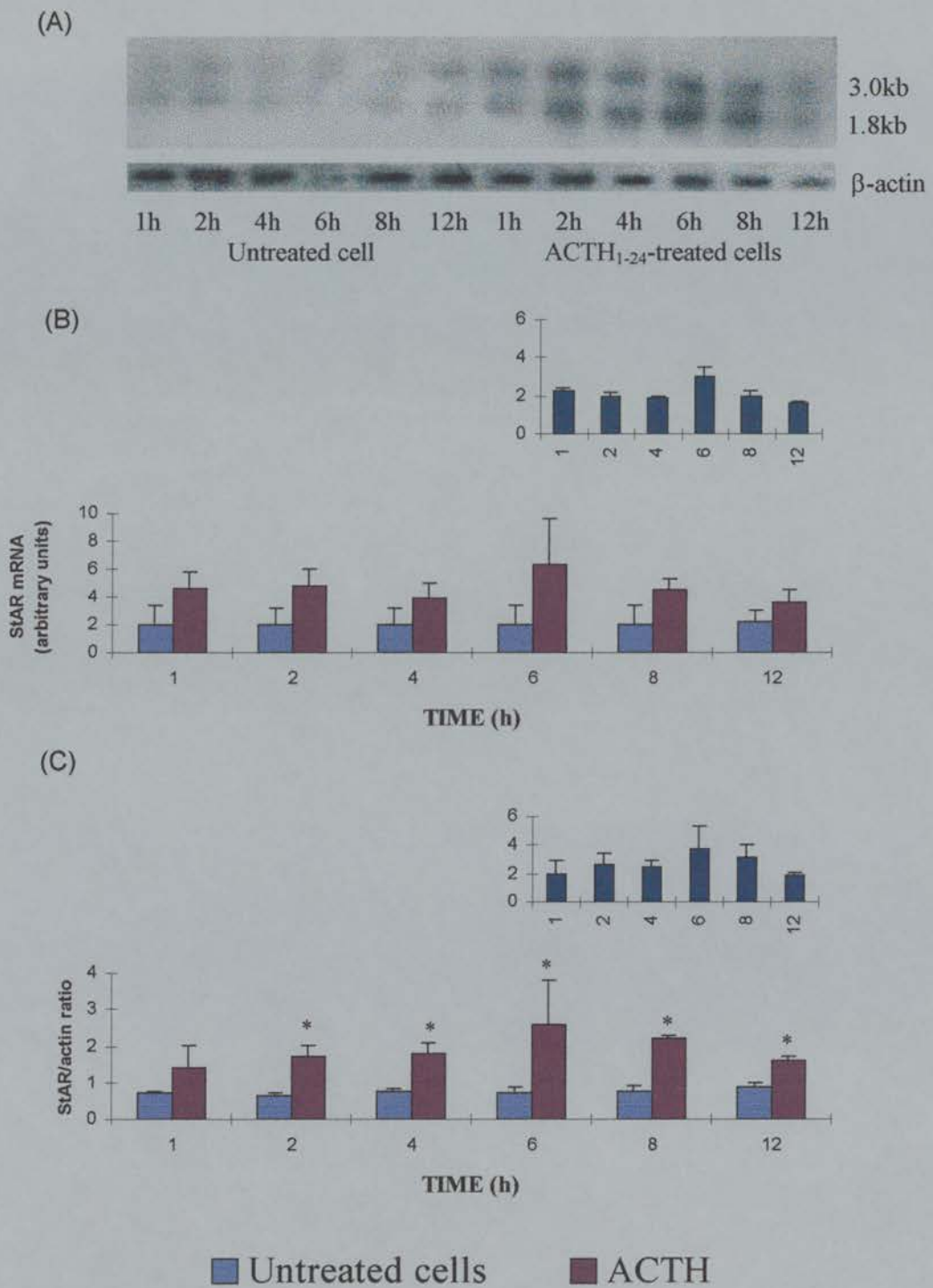


Figure 6.11 StAR mRNA expression in the presence of 5μM cortisone. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) for the times indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts and C: corrected for β-actin. Results are mean ± SD from three independent cell isolations. *$P<0.05$ comparing untreated with $ACTH_{1-24}$-treated cells.

6.2.8 Inhibition of steroidogenesis with etomidate, ketoconazole, trilostane and cycloheximide.

Using a 6 hour incubation the effect of the inhibitors on $ACTH_{1-24}$ treatment of BAC cells was studied. The 6 hour time point was chosen in light of previous results; both cortisol output and StAR mRNA expression are maximal around 6 hours, plus all four steroids were detectable using HPLC at this time.

Figure 6.12 illustrates the cortisol response by BAC cells to $ACTH_{1-24}$ in the presence of etomidate (1&10μM), trilostane (5μg/ml), ketoconazole (5μM) and cycloheximide (100μM). As shown in previous experiments, BAC cells treated with $ACTH_{1-24}$ (10nM) for 6 hours showed a significant increase in the amount of cortisol secreted into the medium overlying the cells when compared with untreated cells ($P<0.001$). The presence of etomidate (1 & 10μM), trilostane, ketoconazole and cycloheximide in the incubation medium significantly reduced the cortisol output in $ACTH_{1-24}$-treated cells ($P<0.001$) (figure 6.12).

No other UV-absorbing steroids were detected in the overlying medium in the presence of etomidate (1 & 10μM), trilostane or cycloheximide (figure 6.13). The peak at around 15 minutes on the etomidate 10μM trace was etomidate itself. There was some cortisol and corticosterone detected in the overlying medium in BAC cells treated with 10nM $ACTH_{1-24}$ plus ketoconazole, 0.7 ±0.3 and 1.3 ±0.3nmol/10^6 cells respectively

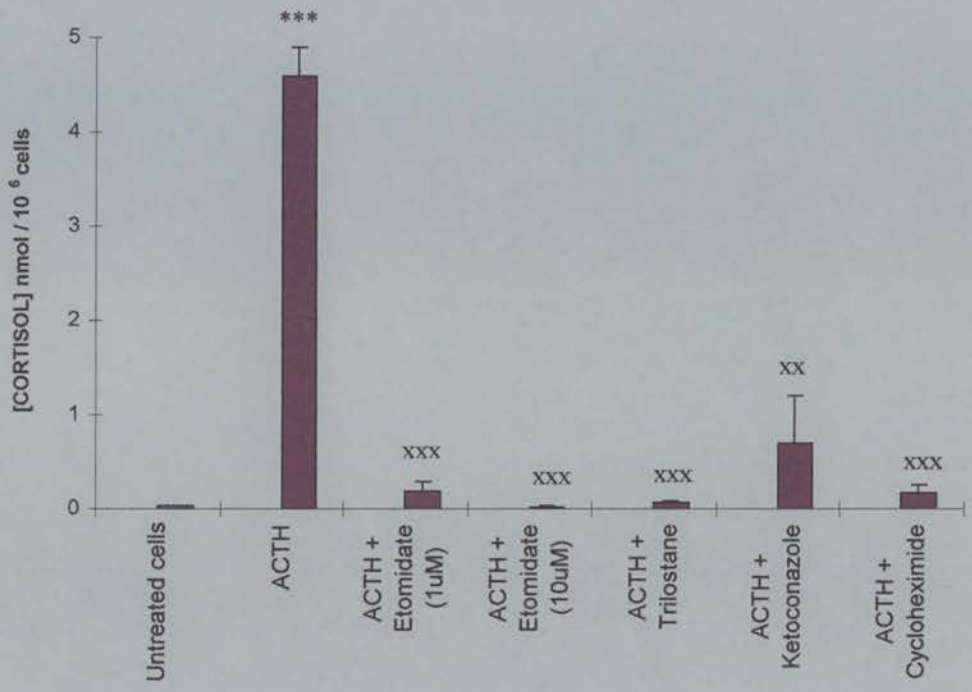


Figure 6.12 Cortisol response by BAC cells treated with $ACTH_{1-24}$ in the presence of inhibitors. BAC were treated with $ACTH_{1-24}$ (10nM) in the presence of etomidate (1 & 10μM), trilostane (5μg/ml), ketoconazole (5μM) and cycloheximide (100μM) for 6 hours. ***P<0.001, comparing $ACTH_{1-24}$-treated cells with untreated cells. xxP<0.01, xxxP<0.001 comparing $ACTH_{1-24}$-treated with $ACTH_{1-24}$ & inhibitors. Result shown are mean ± SD from three independent cell isolations.

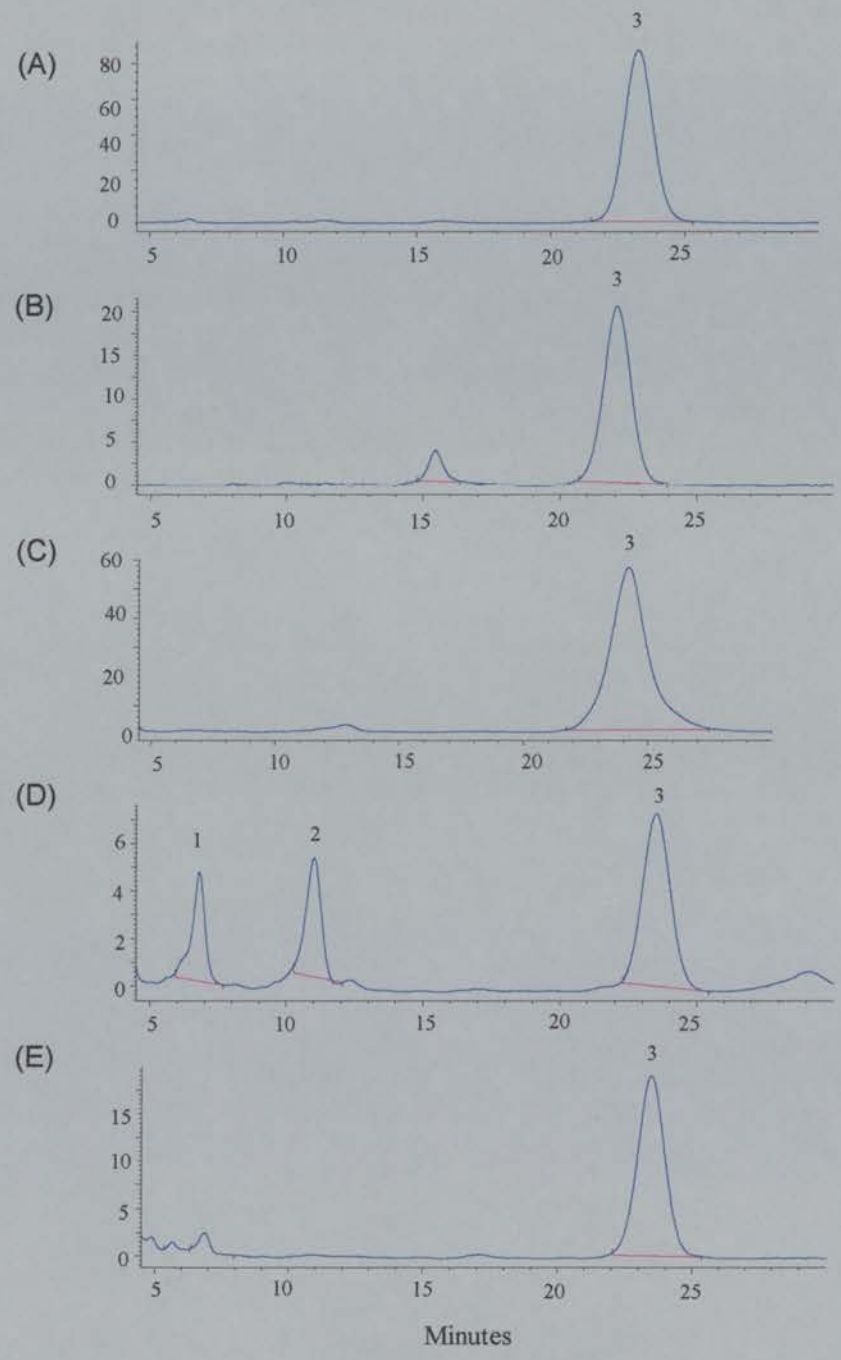


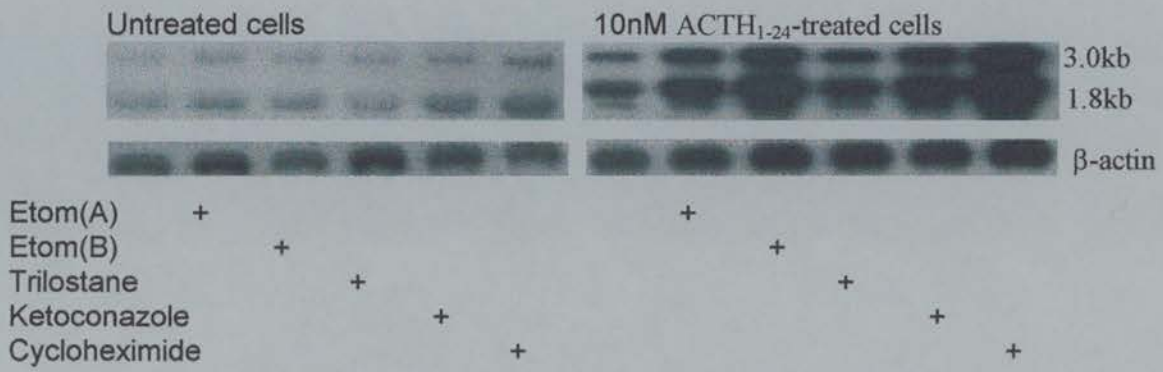
Figure 6.13 Representative HPLC traces of steroids produced by BAC cells treated with $ACTH_{1-24}$ in the presence of inhibitors. BAC cells were treated with $ACTH_{1-24}$ (10nM) in the presence of A & B, etomidate (1 & 10μM), C, trilostane (5μg/ml); D, ketoconazole (5μM), and E, cycloheximide (100μM) for 6 hours. Peak 1 is cortisol; 2 corticosterone and 3 testosterone, the internal standard. The small peak, in trace B, at around 15 minutes is etomidate. Experiments were repeated three times.

6.2.9 Effects of inhibitors on StAR mRNA expression in BAC cells

Study of the StAR transcript expression by northern hybridisation demonstrated an increase in the levels of StAR mRNA in cells treated with 10nM $ACTH_{1-24}$ as seen previously. There also appeared to be an increase in the StAR transcript expression by BAC cells treated with 10nM $ACTH_{1-24}$ in the presence of the inhibitors (figure 6.14). The increase in the StAR transcript levels was most pronounced in the presence of $ACTH_{1-24}$ and cycloheximide, a small increase in the expression of the StAR transcripts in untreated cells in the presence of cycloheximide was also detected.

Quantification of the StAR transcripts showed a significant increase in expression of cells treated with $ACTH_{1-24}$ compared with untreated cells ($P<0.05$). A significant increase in the StAR transcript expression was also observed in $ACTH_{1-24}$-treated cells in the presence of etomidate (1 &10μM), $P<0.05$ and cycloheximide, $P<0.01$, compared with untreated cells. No difference was seen between $ACTH_{1-24}$-treated and untreated cells in the presence of trilostane and ketoconazole (figure 6.15). A 3-fold increase compared with untreated was found in BAC cells treated with 10nM $ACTH_{1-24}$ and $ACTH_{1-24}$ in the presence of 1μM etomidate and trilostane. A 7-, 8- and 10-fold increase compared with untreated was found with ketoconazole, etomidate (10μM) and cycloheximide respectively.

The presence of cycloheximide increased significantly the StAR transcript levels in $ACTH_{1-24}$-treated cells compared to $ACTH_{1-24}$-treatment alone ($P<0.05$). This was not found in the presence of the other inhibitors.



Etom(A):- etomidate (1μM), Etom(B):- etomidate (10μM)

Figure 6.14 Representative northern analysis of StAR mRNA expression in BAC cells treated with or without $ACTH_{1-24}$ (10nM) in the presence of inhibitors. BAC cells were treated as indicated above for 6 hours. RNA was extracted from the cells and using 25μg total RNA/lane subjected to northern hybridisation using a bovine StAR cDNA probe. Two transcripts were found; 3.0 and 1.8 kb. The membrane was stripped and reprobed with β-actin.

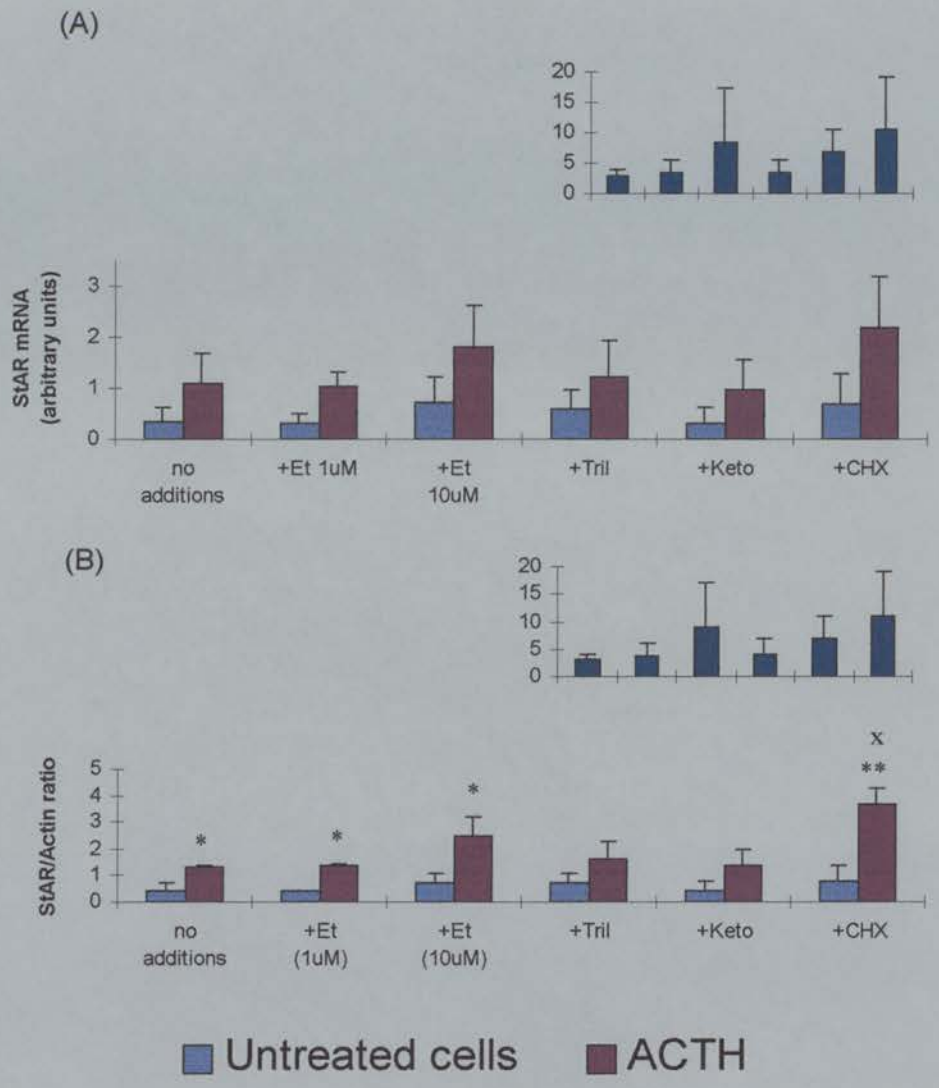


Figure 6.15 Quantification of northern hybridisation. StAR mRNA expression in BAC cells treated with or without 10nM $ACTH_{1-24}$ in the presence of inhibitors. A, sum of the two StAR transcripts shown in figure 6.14. B, the sum of the two StAR transcripts corrected for actin. Mean ± SD from three independent cell isolations. Inserts shown values normalised to untreated cells. *P<0.05, **P<0.01 comparing untreated with ACTH-treated. xP<0.05 comparing $ACTH_{1-24}$-treated with $ACTH_{1-24}$-treated plus inhibitors.
Et, etomidate; Tril, trilostane; Keto, ketoconazole; CHX, cycloheximide.

6.2.10 Effect of etomidate on $ACTH_{1-24}$-treated BAC cells in the presence of steroids.

BAC cells were incubated with or without 10nM $ACTH_{1-24}$ and various combinations of etomidate (1μM), cortisol (5μM) and progesterone (10μM) for 6 hours as follows : 1) with or without $ACTH_{1-24}$ (normal); 2) with or without $ACTH_{1-24}$ plus etomidate; 3) with or without $ACTH_{1-24}$ plus progestrone; 4) with or without $ACTH_{1-24}$ plus progesterone plus etomidate; 5) with or without $ACTH_{1-24}$ plus cortisol; 6) with or without $ACTH_{1-24}$ plus cortisol plus etomidate.

Figure 6.16 displays the cortisol output by BAC cells under these various conditions. BAC cells treated with progesterone, with or without etomidate, produced a significant increase in the amount of cortisol secreted compared with normal untreated cells, $P<0.01$. The presence of etomidate in these cells reduced the cortisol production compared with progesterone alone, $P<0.01$. The addition of cortisol to the culture medium obviously increased the levels in untreated cells compared with normal untreated cells. With the addition of etomidate no decrease in the cortisol levels was observed.

Cells treated with $ACTH_{1-24}$ (normal, cortisol and progesterone) produced a significant increase in the levels of cortisol secreted into the overlying medium compared with corresponding untreated cells, $P<0.01$. When cortisol or progesterone was added to the culture medium BAC cells secreted significantly more cortisol into the overlying medium when treated with $ACTH_{1-24}$ compared with $ACTH_{1-24}$-treated cell alone, $P<0.05$. This increase was found to be additive rather than synergistic compared with the corresponding untreated cells. In the presence of etomidate there was a significant decrease in the amount of $ACTH_{1-24}$-induced cortisol secretion into the overlying medium in all three conditions, $P<0.05$.

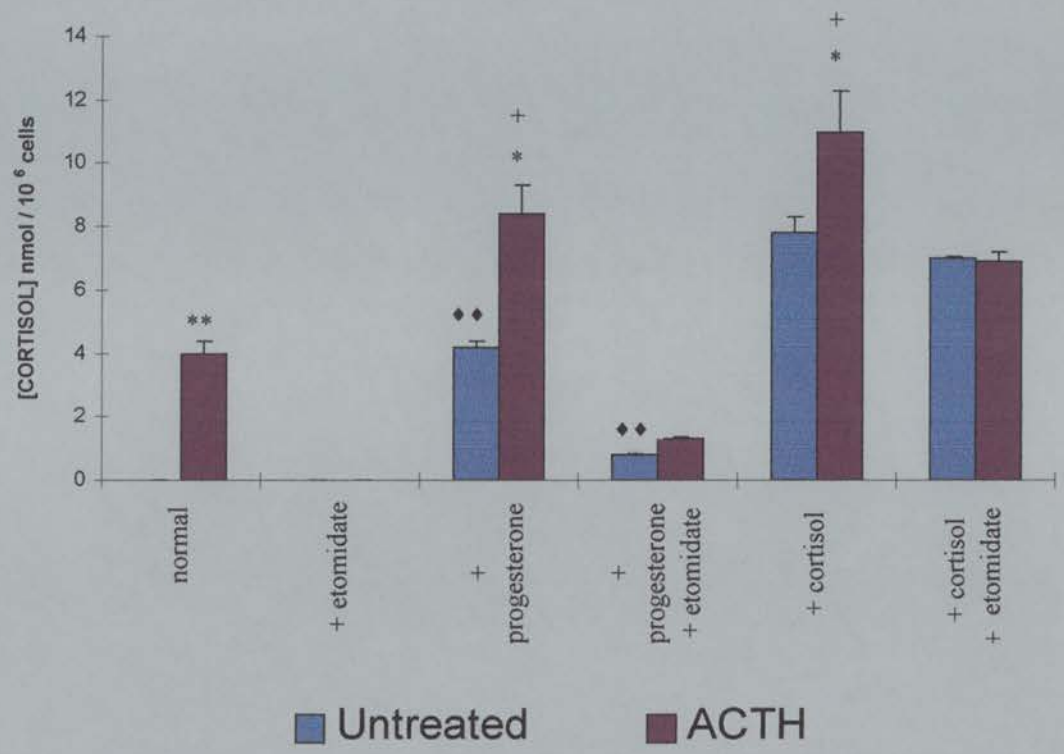


Figure 6. 16 Effect of etomidate (1μM) on the $ACTH_{1-24}$-treatment of BAC cells in the presence of steroids. BAC cells were treated with or without $ACTH_{1-24}$ in the presence or absence of etomidate (1μM), cortisol (5μM) and progesterone (10μM) as indicated. The results are mean ± SD of triplicate wells from one cell isolation. *P<0.05, **P<0.01 comparing untreated with ACTH-treated for each treatment. +P<0.05 comparing normal ACTH-treated with other ACTH-treated. ♦♦P<0.01 comparing untreated (normal) with untreated + additions.

Figure 6.17 displays the northern hybridisation and quantification of the StAR transcript expression by BAC cells treated as described above, this experiment was performed once.

The expression of the StAR transcripts in the northern analysis showed that there was little StAR mRNA expression in untreated cells regardless of the treatment. Upon the addition of 10nM $ACTH_{1-24}$ there was an increase in the levels of the StAR transcripts in all the conditions studied.

Quantification of the northern demonstrated, as previously, the addition of etomidate (1μM) to $ACTH_{1-24}$-treated BAC cells increased the StAR transcript levels compared with $ACTH_{1-24}$-treatment alone. The addition of cortisol (5μM) to the culture medium had no effect on the $ACTH_{1-24}$-induced StAR transcript levels, although the levels were increased by the addition of etomidate. Progesterone increased the StAR transcript levels compared with $ACTH_{1-24}$ alone, with a further increase observed when etomidate was added to the culture medium. The increase in the StAR transcript levels in the presence of progesterone, with and without etomidate, was also found to be greater than that found with ACTH-treatment plus etomidate.

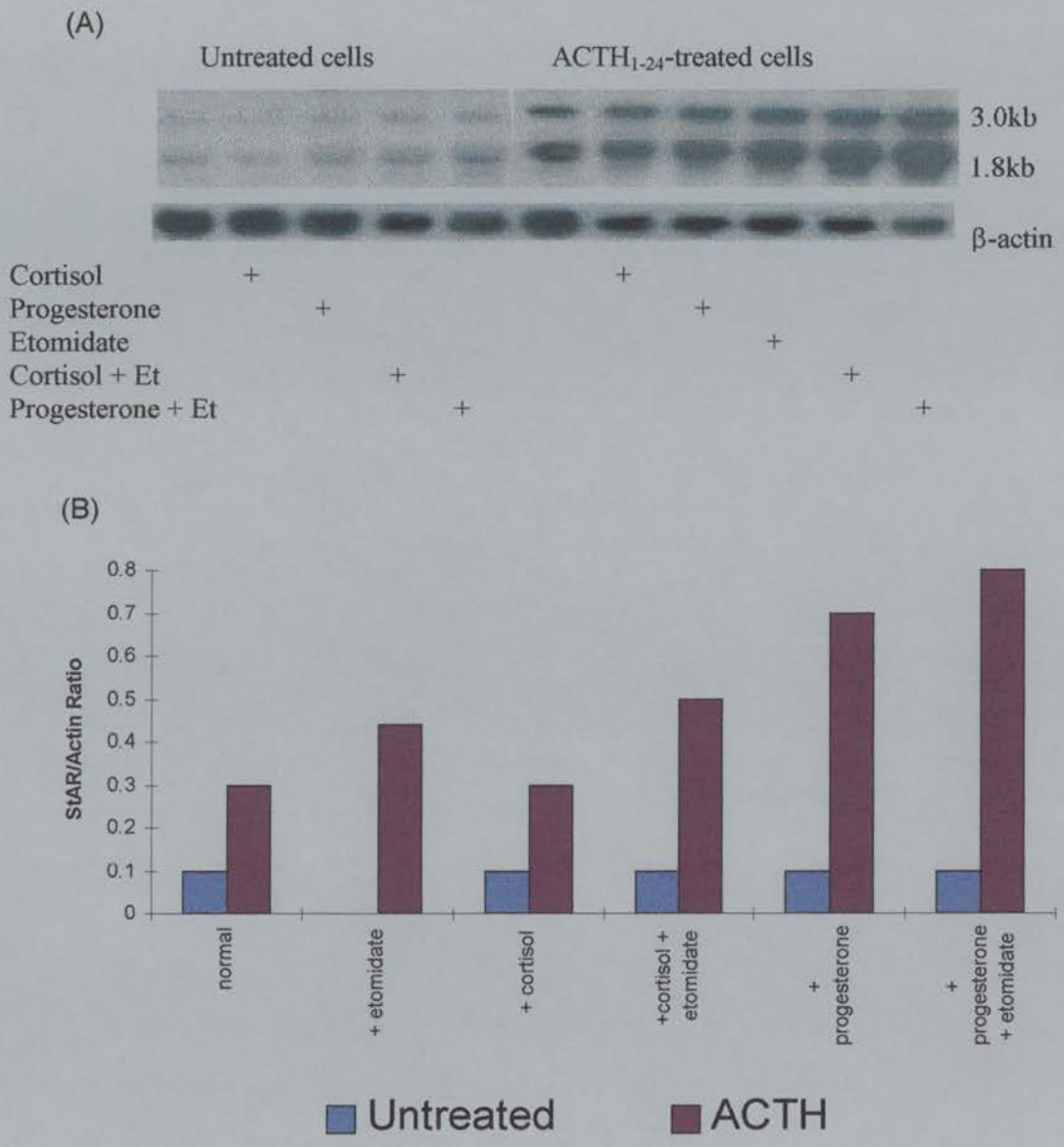


Figure 6.17 StAR mRNA expression in BAC cells treated with $ACTH_{1-24}$ in the presence of etomidate, cortisol and progesterone. BAC cells were treated with or without $ACTH_{1-24}$ (10nM) in the presence or absence of etomidate (1μM), cortisol (5μM) and progesterone (10μM) as indicated. RNA was extracted from the cells and subjected to northern hybridisation using a bovine StAR cDNA probe. A: representative northern analysis showing the 3.0 and 1.8 kb bands. B: quantification of northern hybridisations, shown is the sum of the two transcripts corrected for β-actin. Results are from one cell isolations.

6.3 Discussion

The results presented in chapter 4 demonstrate that when pre-treated with ACTH for 24 hours the cortisol response by BAC cells was comparable to that found in cells without ACTH pre-treatment, indicating that the plateau in cortisol production was not due to ACTH receptor desenitisation. In fact, BAC cells were able to maintain a constant level of cortisol output over a 72 hour treatment period. The results in figure 6.1 show that the response by BAC cells to a second challenge of ACTH is mediated via the transcription of StAR mRNA in a similar manner to the initial ACTH response. Both sets of cell, with or without ACTH pre-treatment, displayed an increase in StAR transcript expression over untreated cells, peaking at around 4-6 hours and declining thereafter to almost untreated levels. This is consistent with in vivo and in vitro studies which have shown that ACTH increases rather than decreases the steroidogenic response to further hormonal stimulation (Penhoat, 1989), (Kolanowski, 1977). In BAC cells, ACTH has been demonstrated to increase the ACTH receptor mRNA after 3 hours of treatment, reaching a maximum by 24 hours (Penhoat, 1995). This would account for the continued response of BAC cells to ACTH seen in this thesis, but does not help explain the plateau in cortisol secretion found after 6 hours of ACTH-treatment.

When the ACTH challenge is renewed every 12 hours, expression of StAR transcripts does not increase above untreated levels whereas a steady level of cortisol output is observed. On reflection, this result is not unexpected as all the ACTH-treatments performed in this thesis have shown a decline in the transcripts to almost untreated levels at 12 hours.

In mammals, ACTH and AngII are the main hormones that regulate steroid secretion by the adrenal gland. AngII has been shown to stimulate steroidogenesis in BAC cells and the results displayed in figure 6. 3 are in keeping with these findings. Although the cortisol response to AngII by BAC cells is about 3-fold less than the

response to ACTH, a similar pattern in the cortisol output is seen i.e. an increase in cortisol output followed by a diminish in production around 4- to 6 hours of AngII-treatment. StAR mRNA expression was found in AngII-treated cells by 1 hour, peaking at around 4 hours and declining thereafter through 12 hours. A similar result was found by Le Roy et al, who showed that both StAR mRNA and protein in response to AngII-treatment of BAC cells peaked within 2-3 hours followed by a rapid decline to almost undetectable levels by 36 hours of treatment (Le Roy, 2000). Thus, indicating that AngII-induced steroidogenesis in BAC cells is also mediated via the regulation of StAR.

The effect of AngII on the StAR transcript expression in ACTH-treated BAC cells was investigated (figure 6.3-5). The cortisol response by BAC cells treated with ACTH and AngII together was also found to be equivalent to that of ACTH on its own. These results indicated that ACTH and Ang II do not act synergistically. Indeed, there was not even an additive effect on the cortisol output. The StAR transcript expression was found to be lower in the cells treated with ACTH and AngII together than in cells treated with ACTH alone. Penhoat et al demonstrated that in BAC cells, AngII caused a decrease in ACTH binding sites and steroidogenic desenitisation (Penhoat, 1995). This would be consistent with the results found in this thesis. A recent study by Le Roy et al showed an strong inhibitory effect of AngII on both cortisol secretion and StAR mRNA expression in BAC cells after 36 hours of AngII-treatment (Le Roy, 2000). Therefore, the inhibitory effect on ACTH-induced cortisol secretion observed in this thesis appears to be StAR-independent, as StAR mRNA levels were still raised during the period studied.

In vivo, ACTH release is regulated by the negative feedback inhibition by cortisol (chapter 1.2). Glucocorticoids, in addition, to their well known effects of inducing a number of genes may act on so-called negative glucocorticoid response elements causing inhibition rather than enhancement of transcription (Bamberger, 1996).

The cell culture system does not posses such a HPA axis. Therefore, to investigate the possibility of a local negative feedback inhibition on steroidogenesis by adrenal steroids BAC cells were incubated with high levels of cortisol or cortisone. The results presented in this chapter show that in the presence of 5μM cortisol, BAC cells were capable of producing cortisol over and above that already in the culture medium, in response to ACTH-treatment. Over the time course studied the cortisol levels of untreated cells decreased with time. HPLC analysis of the culture medium revealed the presence of cortisone in the medium after 4hours ACTH-treatment, previously no steroids were detected in untreated cells. This was probably due to the activity of the 11β-HSD enzyme as a result of the increase levels of cortisol. The levels of cortisone detected in the medium overlying the BAC cells treated with ACTH in the presence of added cortisol were not increased over those found in untreated cells; however the levels of cortisol did not fall to a similar degree found in the untreated cells. This implies that ACTH-treatment was either; 1) increasing the production of cortisol, compensating for the fall found in untreated cells, but not in any quantity to significantly increase the levels over the amount originally found in the culture medium or 2) inhibiting the action of 11β-HSD. ACTH has been demonstrated to lower the capacity of human adrenal slices to convert cortisol to cortisone (Mazzocchi, 1998). The presence of increased levels of cortisol in the culture medium had no effect on the ACTH-induced StAR mRNA expression.

The presence of 5μM cortisone in the cell culture medium had no effect on the ACTH-induced steroid production or StAR transcript expression. This can be explained by the fact that cortisone is an inactive metabolite of cortisol and therefore has no glucocorticoid activity. If the mechanism for the local negative feedback inhibition was via the glucocorticoid receptor then cortisone would have no effect. A study using the mouse adrenal tumor cell line Y-1 demonstrated that corticosterone inhibited ACTH-induced steroidogenesis, whilst other steroids which did not possess glucocorticoid action had no effect. The ACTH receptors in these cells were

unaffected by corticosterone. (Saito, 1979). In rats it was suggested that certain steroids may inhibit adrenal steroid production and that this effect was mediated by inhibition of the synthesis of a regulatory adrenal protein (Morrow, 1967), (Ferguson, 1967). This would be consistent with the hypothesis of a local feedback inhibition of steroidogenesis by adrenal steroids, where the protein inhibited was StAR protein. Nevertheless this was found not to be the case in BAC cells as the addition of cortisol or cortisone to the culture medium had no effect on the ACTH-induced StAR transcript expression.

Dexamethasone has also been shown to inhibit the ACTH-induced accumulation of CYP11A and CYP17 mRNAs at a transcriptional levels in BAC cells. Unpublished observations by this group of researchers have suggested that cortisol, cortisone and corticosterone may also have an inhibitory effect on the accumulation of CYP11A and CYP17 mRNAs in BAC cells (Trzeciak, 1993).

To investigate further the effects of inhibiting the steroid pathway on the expression of the StAR transcripts the protein inhibitor, cycloheximide, and various steroidogenic enzyme inhibitors were used. The effects of cycloheximide on blocking hormone-sensitive steroidogenesis have been well studied (Ferguson, 1962), (Garren, 1965). The results presented in this thesis show the inhibition of steroidogenesis by cycloheximide and that StAR mRNA accumulates within the cells suggesting that the effects of cycloheximide are pre-transcriptional.

The imidazole compounds, etomidate and ketoconazole, were used to inhibit steroidogenesis enzymes post CYP11A, although etomidate at high concentrations can inhibit CYP11A. A greater increase in StAR transcript levels was found with the higher concentration of etomidate. Suggesting that at the lower concentration (1μM) the inhibition was less potent and that some steroidogenesis was occurring although no steroids were found upon HPLC analysis. The measurement of pregnenolone in these samples may have provided useful information. Unfortunately, such an analysis

was not available. Similarly, the increase in StAR transcript levels in the presence of trilostane was less than that observed by the other inhibitors, again the measurement of pregnenolone or 17α-hydroxypregnenolone may have been provide further information. Another imidazole compounds, econazole, has been shown to reduce dbcAMP stimulation of progesterone production in MA-10 cells. In this study econazole increased the expression of StAR mRNA, with a reduction in StAR protein levels observed (Walsh, 2000a). The question as to whether the drugs used in this thesis have the same effect on BAC cell StAR protein levels as found by Walsh et al requires further investigation. The mechanism by which these drugs disrupt the expression of the StAR transcripts remains to be elucidated.

The presence of progesterone in the culture medium produced a significant increase in cortisol production by BAC cells in the absence of ACTH-treatment after 6 hours, with no increase in the expression of StAR transcripts. In the presence of ACTH-treatment a further increase in cortisol levels were found, with a concomitant increase in StAR transcript levels. The increase in StAR mRNA levels was greater than that found with ACTH-treatment alone. The reason for this is unclear at present, as progesterone had no stimulatory effect on StAR mRNA levels in untreated cells and the increase observed in the cortisol output in ACTH-treated cells was additive rather than synergistic compared with untreated cortisol levels. The presence of etomidate in the cell culture medium of both untreated and ACTH-treated cells produced a decrease in the cortisol secreted into the overlying medium, with an increase in the expression of the StAR transcripts found in the ACTH-treated cells. The addition of progesterone to the cell culture medium resulted in a greater increase in ACTH-induced StAR transcript levels, with and without etomidate treatment, than that found with normal ACTH-treatment and etomidate. The reason for this increase is uncertain, also these are the results from one experiment and therefore need to be repeated.

Also in this experiment the addition of cortisol to the cell culture medium produced a significant increase in ACTH-treated cells compared with normal conditions at 6 hours, thus adding to the previous results (section 6.2.5) that cortisol does not exert a negative feedback inhibition in BAC cells. The StAR transcript levels in ACTH-treated cells in the presence of 5μM cortisol was similar to that found in normal ACTH-treated cells, as previously found. The addition of etomidate to the culture medium again decreased the cortisol output with an increase in the StAR transcript levels in ACTH-treated cells.

In conclusion, BAC cells respond to ACTH-treatment with an increase in StAR transcript expression, with a peak in levels found around 6 hours followed by a decline to almost untreated levels by 12 hours. This pattern was found when the cells were subjected to a second ACTH-treatment. Further investigation into the decline in StAR transcript levels and decline in cortisol were undertaken in this chapter. The possibility of cortisol exerting a local negative feedback inhibition was investigated by the addition of cortisol to the medium. However, the result suggested that this was not the case, therefore, cortisone was investigated and was also found not to have any effect on the steroidogenesis of BAC cells. Finally, the use of cycloheximide, trilostane and the imidazole antifungal drugs, etomidate and ketoconazole to help elucidate the effect of inhibiting the steroid pathway on the expression of the StAR transcripts showed that the levels of the transcripts increased in the presence of the inhibitors. Other studies have shown that StAR protein levels are decreased in the presence of other imidazole drugs (Walsh, 2000a), thus suggesting that the process of inhibition, for these drug at least, is post-transcriptional.

Chapter 7 Concluding remarks

The aims of this thesis were to 1) investigate the mechanisms leading to the decrease in the cortisol secretion rate found in BAC cells treated for 12 hours with ACTH, 2) to determine what relationship exits between the pattern of cortisol secretion and the expression of StAR mRNA and 3) to establish whether or not a local feedback inhibition was involved in the secretion of cortisol by cultured BAC cells to account for these findings.

The adrenal cortex is an architecturally complex tissue, with three distinct zones, the zona glomerulosa, fasiculata and reticularis. The steroidogenic output is thought to be dependent on the complex arrangement of the adrenal, determined in part by the flow of steroid precursors from one zone to another (Vinson, 1992), and the proximity of intraadrenal regulatory factors (Ehrhart-Bornstein, 1998). It is of course important to know what products are produced by the whole gland, but it is equally important to know what the contributions are of the individual cell types. *In vitro* studies, whilst not necessarily reflecting the whole physiological situation *in vivo*, are a useful tool for obtaining insight into possible *in vivo* mechanisms. Among studies of adrenal steroidogenesis *in vitro*, methods of tissue preparation have varied widely, from primary cell cultures (DiBartolomeis, 1984), (Walker, 1991), (Williams, 1989) to intact glands(Hinson, 1985), cellular suspensions (Walker, 1991) and tissue slices (Englelhardt, 1991). Isolated cell preparations are an invaluable tool in studying many aspects of cell function and they have been used with great effect in studies to elucidate the mechanism of action of ACTH on glucocorticoid synthesis. The advantages of isolated cells for the study of steroidogenesis include readier access of the trophic hormone to the cell surface receptors and separation of the zones thus determining the actions of each zone individually (Vinson, 1978).

The day of culture on which BAC cells are used for the study of steroidogenesis in culture varies from freshly isolated to cells cultured for up to 6 days, depending on

the researcher (Tachikawa, 1999), (DiBartolomeis, 1984). The first line of investigation in this thesis was to establish the optimum time in culture to study the cortisol response by BAC cells. The results presented here clearly establish that BAC cells were less responsive to ACTH after isolation but recover with time in culture. The cells secreted cortisol in response to ACTH on each of the days studied, but were less able to maintain the levels of cortisol output over a 24 hour period in the freshly isolated cells. Possible reasons may the reduced lipid content or a reduction in the number of functional ACTH receptors (Williams, 1989), (Penhoat, 1989). The response by BAC cells to ACTH-treatment was found to be maximal on day 3 of culture, thus further experiments were carried out at this time.

Previous studies have investigated the short-term (up to 6 hours) effects of ACTH on the acute steroidogenic mechanisms in adrenocortical cells (e.g. the second messenger responses) or have investigated the longer-term (48 hours or over) effects, (e.g. induction of the steroidogenic enzymes) (Clyne, 1993), (Walker, 1991), (DuBois, 1981), (Funkenstein, 1983). The work carried out in this thesis investigates the responsiveness of BAC cells to ACTH-treatment at the intermediate time periods.

The cortisol output by BAC cells treated with ACTH (10nM) was found to increase over the initial 6 hours with a decrease in the cortisol secretion rate between 6 and 12 hours. The protein content of the culture wells was measured to ensure that the decline in cortisol secretion was not due to the detachment of cells. This was found to be constant over the 12 hour time period. Several lines of investigation were undertaken to investigate the decline in cortisol secretion observed in BAC cells.

Cholesterol is essential for cortisol production and is supplied to the steroidogenic cells either via *de novo* synthesis or from exogenous sources such as HDL or LDL (Krieger, 1999). Serum is essential in the initial stages of primary culture for effective plating and maintenance of the cells. However, when studying the steroidogenic response of BAC cells to various agonists it is advantageous to use a serum-free

medium. thus ensuring the responses found are due to the agonists used and not as a result of hormones or other substances found in the serum (Hornsby, 1991). The results presented in figures 3.3 demonstrate that ACTH produced a comparable cortisol responses under both lipid-deplete and lipid-replete conditions. A previous study also demonstrated that BAC cells grown in the absence of exogenous cholesterol can produce steroids in response to ACTH by increasing their ability to synthesise cholesterol *de novo* (Rainey, 1986). This, argues against the decline in steroidogenesis seen in the BAC cells as a result of a lack of exogenous cholesterol supply.

The profile of steroids produced by BAC cells in response to ACTH-treatment was investigated to determine whether the decline in cortisol output was due to other steroids being synthesised in preference to cortisol (e.g. corticosterone). Another possibility may be the conversion of cortisol to its inactive metabolite cortisone, as 11β-HSD activity has been found in BAC cells (Romero, 2000). Using HPLC, four principal steroids were detected in the medium overlying BAC cells; cortisol, corticosterone, 11 β-hydroxyandrostenedione and cortisone. Over the initial 4 hours of ACTH-treatment only cortisol and corticosterone were detected, both being produced in about equal amounts for the first 2 hours of treatment. Cortisone and 11β-Hydroxyandrostenedione were not detected in the medium overlying the BAC cells until 6 hours of ACTH-treatment. After the initial 2 hours, cortisol was found to be the main steroid (≥50%) produced at each of the time points studied. The amount of cortisone in the medium overlying the BAC cells did not increase significantly over the time period studied and failed to account for the decrease in cortisol output. In fact the total steroid production (figure 3.7/table 3.1) displayed a decrease in production after 6 hours.

The decline in the rate of cortisol secretion was also found over a range of ACTH concentrations, with the plateau most apparent with the lower concentrations of ACTH used. ACTH 1nM produced a plateau in cortisol output between 4 and 6

hours, whereas with 100nM ACTH the plateau was found to occur between 8 and 12 hours. $ACTH_{1-24}$ (synacthen) was used in these experiments, as an alternative to the native $ACTH_{1-39}$ peptide. In most species studied the N-terminal 24 amino acids are identical and this is the part essential for adrenocortical activity. (Schwyzer, 1977). To determine if the decrease in cortisol secretion was due to some inherent property of the truncated analog, BAC cells were treated with the $ACTH_{1-39}$ peptide. No difference in the cortisol response compared with $ACTH_{1-24}$ was found. These findings are important in that they demonstrate the particular pattern of secretion in response to $ACTH_{1-24}$ is comparable to native $ACTH_{1-24}$.

In order to determine if this decline in steroidogenesis was confined to ACTH, the effect of other agonists on cortisol production over the 12 hour time course was also studied. The data presented in this thesis demonstrated that 8Br-cAMP and forskolin increased cortisol production significantly compared with untreated cells ($P<0.05$) at all of the time points studied. An interesting observation was that no plateau in cortisol production was observed with forskolin or 8Br-cAMP. HPLC analysis indicated that the four main steroids were produced by forskolin, in a similar pattern to that found with ACTH. In response to 8Br-cAMP cortisol, corticosterone and cortisone were detected but no 11β-hydroxyandrostenedione was found, possibly due to the lower overall level of steroids produced by the BAC cells treated with 8Br-cAMP. This suggests that the decline in cortisol production was possibly specific to ACTH related to an event pre-cAMP as both forskolin and 8Br-cAMP utilise the cAMP second messenger system as well as ACTH. Nevertheless, the measurement of cAMP levels may have provided useful information as it has been shown that ACTH increases the levels of cAMP in BAC cells (Walker, 1991).

To determine if the decline in cortisol secretion was found with an alternate second messenger pathway AngII was used to treat the cells. AngII acutely increases aldosterone synthesis in bovine glomerulosa cells through the activation of PKC and calcium second messenger pathways (Kojima, 1985). An increase in cortisol output

was found in AngII-treated BAC cells (figure 3.14), results which are consistent with previous studies (Bird, 1989), (Clyne, 1993), (Le Roy, 2000). The data presented in this thesis demonstrated that AngII produced a significant increase in cortisol production over untreated cells at all of the time points studied, with a plateau in cortisol production at 4 hours of treatment.

The decline in steroidogenesis was therefore not limited to ACTH-treatment of BAC cells. Since the actions of both ACTH and AngII are mediated through cell surface receptors (whereas forskolin and 8Br-cAMP are not). A further possibility is that the decline in cortisol output seen in BAC cells could be through receptor desensitisation. The initial phase of receptor occupancy and target cell activation is frequently followed by a period of diminished responsiveness. This may be due to the presence of residual hormone on the receptor site (Catt, 1979).

BAC cells treated for 24 hours with ACTH (10nM) and then re-challenged with ACTH (10nM) for the 12 hour time course showed a comparable response to those treated with ACTH without pre-treatment. It therefore appears that the BAC cells did not become desensitised to ACTH with continued exposure to ACTH-treatment. Indeed, the cells pre-treated with ACTH for 24 hours, showed an increase in the amount of cortisol secreted compared with those without pre-treatment, (significant at 8 and 12 hours, $P<0.05$). This might be explained as the result of the up-regulation of various steroidogenic enzymes with the 24 hour ACTH-pre-treatment (Simpson, 1988), or due to an increase in the number of ACTH receptors. Various studies on the ACTH receptor have shown that ACTH has a positive effect on its own receptor, (Penhoat, 1995), (Penhoat, 1994), (Liakos, 1998). In BAC cells, 10nM ACTH-treatment was found to maximally increase the ACTH receptor mRNA after 24 hours. This stimulatory effect of ACTH on the ACTH-R mRNA was associated with an increase in receptor numbers (Penhoat, 1995).

Studies performed in this thesis also demonstrated that BAC cells could maintain a level of cortisol output over a 72 hour time period. BAC cells treated with ACTH which was renewed every 12 hours throughout 72 hours displayed a consistent repetitive pattern of cortisol output. Further experiments to investigate whether the cortisol response over each of the 12 hour challenges (1-12 hour treatments) was similar to that seen originally may have been more informative. Overall these results corroborate the earlier studies on the use of serum-free medium for the experiments. Thus, BAC cells were able to maintain a steroidogenic output after 24 hours ACTH treatment or up to 72 hours ACTH treatment in serum-free medium, implying that the cells have an adequate supple of cholesterol, possibly from *de novo* synthesis (Rainey, 1986).

The stability of the ACTH also investigated. $ACTH_{1-24}$ alone was incubated overnight at 37°C and the following morning placed on BAC cells. The cortisol output was compared with that found for ACTH prepared freshly on the day of the experiment. At the lower concentrations used (0.01 and 1nM) there was a significantly reduced response in cortisol production compared with ACTH prepared on the day of the experiment. The two higher concentrations (10 and 100nM) showed no difference. In order to investigate of the stability of ACTH, the concentration in the cell culture medium was measured. $ACTH_{1-39}$ was used in these experiments due to the immunometric assay used. $ACTH_{1-39}$ was incubated overnight at 37°C in tissue culture plates without BAC cells present. The concentration of 1nM ACTH was reduced to 0.1nM, whereas 10nM and 100nM were reduced to 2.7 and 61nM respectively (figure 4.4). These results show that although 10 and 100nM ACTH are reduced significantly in concentration the final concentration is still sufficient to elicit a cortisol response. When the ACTH was incubated overnight in the presence of BAC cells, the fall in concentration of ACTH (10nM) was less dramatic compared with the ACTH incubated in the absence of BAC cells (figure 4.7). These finding provide only a partial explanation for the decline in the cortisol output observed in BAC cells, since the studies presented here were carried out using 10nM ACTH. The decrease in the

immunoactivity of ACTH especially at the lower concentrations, contributes to the decline in cortisol secretion. The reason for the observed decline in all of the ACTH concentrations in the absence of cells is unclear, though the hypothesis that ACTH binds to plastic may provide an answer. This may also help explain the plateau observed with AngII-treatment, although this line of investigation was not followed in this thesis.

The next approach was to look at the expression of StAR mRNA to determine if there was a relationship between cortisol secretion and StAR mRNA expression. StAR protein mediates the delivery of cholesterol into the mitochondria where it is converted to pregnenolone by CYP11A (Orth, 1992). StAR mRNA is regulated by trophic hormone in a time frame concomitant the acute production of steroid hormones (Stocco, 1991), (Clark, 1994). The initial experiments performed in the thesis were to characterise the expression of StAR mRNA in BAC cell culture. On northern hybridisation of the StAR mRNA two transcripts were found, 3.0 and 1.8kb, consistent with other studies (Ivell, 2000), (Le Roy, 2000). The first step in the study of StAR mRNA expression in BAC cells was to measure the response on day 3 since previous experiments had shown an enhanced cortisol response on this day. Both cortisol output and StAR mRNA expression in response to ACTH-treatment were found to be maximal on day 3 of culture.

The StAR mRNA expression in BAC cells over a 12 hour period followed a similar pattern to cortisol. There was an increase in expression over the initial 4-6 hours, peaking at around 4 or 6 hours, followed by a decrease in StAR transcript levels to almost untreated levels by 12 hours of ACTH-treatment. This pattern was seen with various concentrations of ACTH. At 1pM ACTH the cortisol response and the levels of StAR mRNA did not correlate. A significant increase in cortisol was seen over the 6 hour treatment but no increase in StAR mRNA levels was detectable (figures 5.4&5.5). A previous study has demonstrated that cortisol secretion and StAR protein expression differ in response to high (10nM) versus low (1pM) ACTH-treatment in

BAC cells (Wang, 1999), with a strong correlation between cortisol secretion and StAR protein induction at the higher but not at the lower ACTH concentrations. Increasing evidence has suggested that arachidonic acid metabolites may be important second messengers for ACTH stimulation (Yamazaki, 1996), (Finkielstein, 1998). A study by Wang et al has demonstrated that in BAC cells, ACTH-treatment at low concentrations is mediated via arachidonic acid metabolites involving a StAR-independent mechanism (Wang, 2000).

Forskolin and 8Br-cAMP also increased the expression of StAR mRNA. In response to forskolin a peak in StAR mRNA levels at around 4 hours were observed; however the levels did not fall to near untreated levels by 12 hours but remained elevated above untreated values, ($P<0.05$ at 8 hours). No peak in StAR mRNA levels was found with 8 Br-cAMP. Instead a constant level of expression, above that found in untreated cells, was seen over the 12 hour time period. This was inconsistent with other studies. In MA-10 cells Clark et al demonstrated that the levels of StAR mRNA in response to dbcAMP peaked at around 6 hours of treatment followed by a decrease in levels by 12 hours (Clark, 1995). The reason for this discrepancy is unclear at present. Both the cortisol output and the StAR mRNA expression in BAC cells treated with forskolin and 8Br-cAMP remain elevated over the 12 hour time period. This also accords with the ongoing cortisol response, as distinct from the decline in secretion rate observed with ACTH-treatment.

After treatment of BAC cells with AngII there was an increase in the relative amounts of the two StAR transcripts. The increase was already detectable after 1 hour of treatment, by comparison with the untreated levels, and reached a maximum at about 4 hours. As with ACTH-treatment there was a rapid decline in transcript levels thereafter. AngII has also been shown to acutely stimulate StAR protein in bovine zona glomerulosa cells (Brand, 1998), (Elliott, 1993). Thus, the induction of StAR mRNA in response to ACTH, forskolin, 8Br-cAMP and AngII argue that cAMP- or

protein kinase C-dependent processes may be key regulators of StAR mRNA expression.

As discussed earlier, BAC cells pre-treated for 24 hours with ACTH prior to a second 12 hour ACTH-treatment showed a repeat of the pattern of secretion seen without pre-treatment. The next step was to determine if this second increase in the cortisol response was mediated via an second increase in StAR transcription. BAC cells treated for 24 hours with ACTH (10nM) and then re-challenged with ACTH for the 12 hour time course showed a comparable StAR mRNA response to those treated with ACTH and no pre-treatment. Therefore the second ACTH-treatment was also mediated via the up-regulation of StAR transcription, suggesting the start of a new cycle of steroid production rather than a continuation of the first one.

Previous studies have provided some evidence that certain steroids may inhibit adrenal steroid production and that this may be mediated by the inhibition of adrenal protein synthesis (Morrow, 1967), (Ferguson, 1967). Therefore, an obvious candidate for this inhibitory factor in the medium could be cortisol itself. *In vivo,* cortisol is produced by the adrenal cortex under the regulatory influence of ACTH, which in turn is under the influence of CRH (see section1.2). The HPA axis is kept in balance by the negative feedback effects of cortisol on the secretion of ACTH and CRH (Aron, 2000). At the cellular level, the effects of cortisol are mediated via the glucocorticoid receptor (GR) (Bamberger, 1996). Hormone binding to the GR activates the receptor causing it to bind to GREs in the regulatory region of target genes (see section 1.4.5). This interaction causes either stimulation or, less frequently, inhibition of transcription (Bamberger, 1996). One important example of negative regulation by the GR is the feedback inhibition mechanism of the HPA axis (Reichardt, 1998). Glucocorticoids themselves appear to be potent regulators of glucocorticoid receptor expression and have been shown to cause down-regulation of the receptor in many cell lines and in tissues or cells from intact animals and healthy human subjects (Burnstein, 1992).

To test the hypothesis that cortisol was exerting a local negative feedback inhibition within the BAC cell culture, cortisol (5μM) was added to the culture medium. At 0 hour the level of cortisol in the cell culture medium was measured as 5.5 ±0.5nmol/10^6 cells. The addition of ACTH to the cells resulted in an increase in the cortisol levels, although this was found to be significant only at 4 and 8 hours. The expression of the StAR transcripts in the presence of increased cortisol levels was found to be comparable with that found under normal conditions. These experiments do not support the hypothesis that increased cortisol levels in the medium exert any major negative feedback effect on the steroidogenic capability of BAC cells.

Although cortisone is metabolically inactive and the levels of cortisone did not exceed those of cortisol, the possibility this steroid was exerting a negative effect on the steroidogenic capability of the cells was also investigated. The cortisone levels in the untreated cells were found to decrease with time. The reason for this is unclear since the metabolism of cortisone normally occurs in the liver (Orth, 1992). When BAC cells were treated with ACTH in the presence of increased levels of cortisone a significant increase in cortisol output was found at each of the time points studied. Moreover, the presence of cortisone appeared to have a slight enhancing effect on the cortisol output of the BAC cells, although no effect on the ACTH-induced StAR mRNA expression was found. ACTH has been shown to lower the conversion of cortisol to cortisone in human adrenal slices (Mazzocchi, 1998). One explanation for the enhanced cortisol secretion could be that due to the raised levels of cortisone this conversion is depressed further with less newly synthesised cortisol converted to cortisone.

The decrease in cortisol output observed in cultured BAC cells was not due to the presence of cortisone accumulation in the medium and was unlikely to be due to the increasing levels of cortisol. Further studies on different steroids, e.g. corticosterone,

in the cell culture medium may have been informative. The preffered diversion of pregnenolone into the synthesis of the cardiotonic steroid ouabain may provide a further possibility. Ouabain is a plant derived cardiac glycoside with Na^+-K^+-ATPase inhibitory properties (Doris, 1994). Ouabain or a 'ouabain-like compound' (OLC) has been detected in rat, bovine (Doris, 1989) and human (Hamlyn, 1991) adrenal gland and in the culture medium of bovine adrenocortical cells (Laredo, 1995), (Perrin, 1997). The biosynthetic pathway of this compound in mammals and specifically in adrenocortical tissue is unclear. Cardiac glycosides possess a basic steroid skeleton and in plants it has been shown that cholesterol, pregnenolone and progesterone are metabolic precursors (Doris, 1994). The presence of cholesterol from endogenous or exogenous sources, and of pregnenolone and progesterone as intermediates suggests a possible biosynthetic pathway in the adrenal. Incubation of BAC cells with excess pregnenolone or progesterone increased the production of OLC (Perrin, 1997). One possibility could be that at the later time points (6-12hours) the BAC cells were producing OLC in preference to cortisol. The measurement of OLC in the culture medium overlying the BAC cells used in this thesis may have shed light on this. However, there have been discrepancies in the measurement of ouabain, by either HPLC or RIA, with some researchers unable to detect OLC (Doris, 1994). This line of investigation was not followed; nevertheless, it is interesting that both ACTH and AngII have been shown to stimulate the secretion of OLC by BAC cells (Laredo, 1995).

The use of ACTH-conditioned-medium on the BAC cells suggested that one or more factors were secreted into the medium overlying the cells which might inhibit further steroidogenesis. BAC cells treated with ACTH-conditioned medium (figure 4.9) showed no significant increase in cortisol output above that already found in the ACTH-conditioned medium alone. The ACTH already in the conditioned medium did not stimulate the untreated, set B cells. When further ACTH was added to the medium there appeared to be some increase over the ACTH-conditioned medium alone; however this trend was found to statistically insignificant from the 0h level.

These results suggest that there may be one or more factors secreted into medium that attenuate the ACTH-induced cortisol production in BAC cell culture but may be degraded over time, thus leading to the statistically insignificant trend in cortisol. The expression of StAR transcripts in the conditioned medium conditions would have been of potential interest and might help to resolve the question as to whether steroidogenesis was actually inhibited under these conditions or not. These experiments were not done due to a lack of time.

It is generally accepted that glucocorticoids act indirectly on the adrenal cortex by inhibiting the HPA axis (Aron, 2000). The existence of a direct inhibitory effect of glucocorticoids on the adrenocortical cells is more controversial. Some *in vivo* studies (Hill, 1968), (Black, 1961) and *in vitro* (Salmenpera, 1976), (Morrow, 1967) have suggested a suppressive effect by glucocorticoids on adrenal steroidogenesis. In this connection, evidence has been obtained that adrenocortical cells possess glucocorticoid receptors (GR) (Loose, 1980) and that glucocorticoids themselves appear to be potent regulators of these receptors (Burnstein, 1991). It is possible that the negative effect of glucocorticoids on GR expression represents a feedback inhibition mechanism. This mechanism may be important *in vivo* for protecting the adrenal from excessive glucocorticoid levels in states of hypercorticoidism.

In conclusion, the main steroid produced by BAC cells in response to ACTH-treatment was cortisol. The response by BAC cells to ACTH-treatment was an increase in cortisol output over the first 6 hours followed by a plateau in cortisol output thereafter. The output in cortisol was mirrored by an increase in StAR mRNA expression, with a peak in StAR mRNA expression at 6 hours followed by a decline thereafter. Thus showing a temporal relationship between steroid production and StAR mRNA expression in BAC cells. A series of experiments to investigate the cause of this decline in steroidogenesis were undertaken. Various aspects of the cell culture system, such as serum, were looked at and found not to influence either steroid output or StAR mRNA expression. The concentration of ACTH and the

peptide length were found not to be a factor. The decline in StAR mRNA and cortisol production was found with various ACTH concentrations and with both $ACTH_{1-24}$ and $ACTH_{1-39}$. The bioactivity of ACTH was reduced after 24 hours in the cell culture system at the lower concentrations of ACTH. The binding of ACTH to the plastic of the cell culture plates was a possibility; this could be investigated further by using radiolabelled ACTH. Desensitisation of the ACTH receptor was investigated by retreating the BAC cells with ACTH after 24 hours. The steroid output and StAR mRNA expression in BAC cells treated with ACTH for a second time was similar to that seem with the first ACTH treatment. This suggests that the ACTH receptor was fully functional.

The use of conditioned medium suggested the presence of a factor(s) within the cell culture system that could be having an inhibitory effect on steroidogenesis. One possibility was that the high level of cortisol secreted into the medium was exerting negative feedback inhibition. However this was found not to be the case when cortisol added to the cell culture medium had no effect on either the steroid output or the StAR mRNA expression in ACTH-treated BAC cells. Further studies into the identification of the proposed inhibitory factor would be interesting. However, this may prove to be a laborious task as many factors are secreted into the cell culture medium. The decline in steroidogenesis may also be the result of the "static" nature of the cell culture system. Further experiments using a superfusion system whereby the medium bathing the cells is continually moving, therefore more akin to the situation *in vivo*, would be of interest.

Another possibility is that glucocorticoids may act negatively to regulate gene expression of the steroidogenic enzymes, although this was not investigated in this thesis. It has been shown that the glucocorticoid dexamethasone can suppress the ACTH-induced accumulation of CYP11A and CYP17 mRNAs with a concomitant fall in cortisol secretion (Trzaciak 1993). Measurement of the mRNA levels for the various steroidogenic enzymes throughout the ACTH treatment period would be of

interest. This could be done using northern analysis or real-time RT-PCR. Developments in DNA microarray technology, which allows patterns of gene expression of several steroidogenic enzymes to be analysed at once, would be beneficial for further studies in this area.

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NICOL, M.R., WANG, H., IVELL, R., MORLEY, S.D., WALKER, S.W. AND MASON, J.I. **1998** The expression of steroidogenic acute regulatory protein (StAR) in bovine adrenocortical cells. *Endocrine Research* 24:565-569.

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CONFERENCE PRESENTATIONS

NICOL, M.R., MORLEY, S.D., STIRLING, D., IVELL, R., WALKER, S.W., AND MASON, J.I. **1997** The expression of steroidogenic acute regulatory protein (StAR) mRNA in bovine adrenocortical cells. *Journal of Endocrinology* 152 (suppl) P7.

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THE EXPRESSION OF STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) IN BOVINE ADRENOCORTICAL CELLS

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ABSTRACT

StAR protein may facilitate rapid transfer of cholesterol from the outer to the inner mitochondrial membrane, the site at which cholesterol is converted to pregnenolone by the cholesterol side chain cleavage complex. We have studied the effect of ACTH treatment on StAR mRNA and protein levels in bovine adrenocortical cells in primary culture. Cells were initially cultured for 3 days after isolation, and then treated with ACTH (10^{-8} M) for various times up to 24 hours. Northern analysis of total BAC mRNA, using a [α^{32}P]-labelled cDNA probe encoding a 5′ region of bovine StAR mRNA, revealed two principal hybridising species of 1.6 and 3.0 kb. Western immunoblot analysis revealed a principal band at 30 kDa. Levels of both StAR mRNA and protein showed an increase at 1 hour, reached a maximum at around 6 hours and declined to basal levels at 24 hours. Cortisol secretion (measured by RIA) showed a similar change over the same period. From these results it appears that StAR mRNA and protein levels in BAC are acutely regulated in concert with ACTH-stimulated cortisol secretion.

INTRODUCTION

The first enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone ,catalysed by the cytochrome P450 side chain cleavage (P450scc) enzyme which resides on the inner mitochondrial membrane. The rate-limiting step in this process is the translocation of cholesterol from the outer to the inner mitochondrial membrane, which requires a rapidly synthesised regulatory protein. StAR protein was initially described in rat and bovine adrenal cells by using two–dimensional gel

electrophoresis to identify a family of mitochondrial proteins synthesised in response to ACTH-stimulation. StAR protein has also been found in other steroidogenic tissues (1,2) and StAR cDNA was first cloned and sequenced from mouse MA-10 Leydig tumour cells (3). MA-10 and COS-1 cells transfection with StAR cDNA, in conjunction with the P450scc system, resulted in steroidogenesis in the absence of trophic hormone stimulation (4,5). Demonstration that the inherited defect, lipoid congenital adrenal hyperplasia, is due to mutations in the StAR gene further emphasises the its role in steroidogenesis (6). StAR cDNAs have now been cloned from a variety of tissues and species including bovine luteal cells where the corresponding mRNA was found to be transcribed as 3 kb and 1.6 kb transcripts (7). In this study, we have examined the regulation of StAR mRNA and protein levels in bovine adrenocortical (BAC) cells in response to trophic hormone treatment.

METHODS

BAC cells were isolated and cultured as previously described (8). Cells were plated at a density of 5 x 10^6 per 25 cm^2 flask for mRNA studies and 1.5 x 10^6 per well of a 6-well plate for protein studies. Total RNA (25 µg) was analysed by northern blot and StAR mRNA was detected using a [α^{32}P]-labelled cDNA probe encoding a 5' region of the bovine StAR (GenBank acc.# S79908, position 210-608). Total cellular protein (25 µg) was analysed by western blot using a primary polyclonal sheep antiserum raised against a peptide fragment (amino acids 82–107) of the bovine StAR protein and a donkey anti-sheep second antibody coupled to a HRP-conjugate (The Binding Site, Birmingham, UK). The final signal was detected by chemiluminescence (SuperSignal ULTRA kit, Pierce, Rockford, USA). Cortisol contents of media were quantified using a double antibody radioimmumoassay.

RESULTS AND DISCUSSION

This study demonstrates the presence, in BAC cells, of two principal StAR mRNA transcripts of 1.6 and 3.0 kb and principal and minor protein species of 30 and 40 kDa, similar to other studies on StAR mRNA (9) and protein (10) in BAC cells. Distinctly in the present study, western blot analysis shows that freshly isolated BAC cells contain

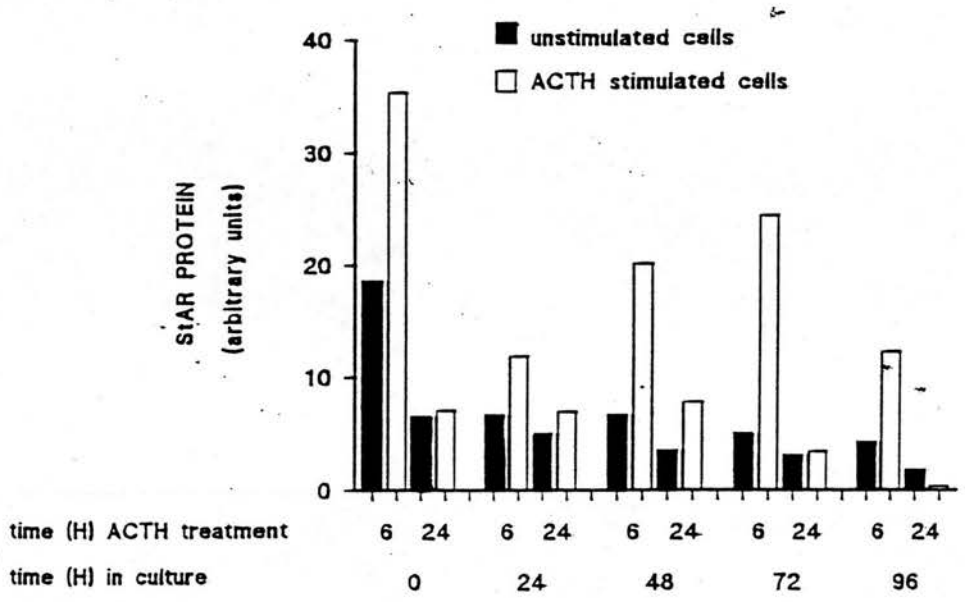

FIGURE 1

BAC cells isolated at time 0 were cultured for 24, 48, 72 or 96 hours and then treated with/without ACTH (10^{-8} M) for a further 6 and 24 hours. Quantification of Western blot analysis (30 kDa band) was made using an image analyser.

high levels of StAR protein, while these levels decrease over 3 days of culture. ACTH treatment for 6 hours following 24, 48, 72 or 96 hours of culture increases the StAR protein levels; however, when ACTH treatment is prolonged for 24 hours, the StAR protein levels return to untreated levels (Figure 1). Western blotting and northern analysis show that StAR gene expression appears to be most responsive to ACTH stimulation following 48 and 72 hours of culture (Figures 1 and 2). We therefore chose to use cells cultured for 72 hours for subsequent experiments.

Following ACTH treatment StAR mRNA levels and cortisol secretion were increased after I hour (Figures 2B and 3), whereas StAR protein levels were not increased above that in untreated cells until 6 hours of treatment (Figure 2A). This suggests that the initial steroidogenic response is not primarily dependent on the presence of high levels of newly synthesised StAR protein, although participation of a small sub-pool of new protein cannot be excluded. Instead, mechanisms, such as post-translational modification of an existing pool of StAR protein, probably initiate the initial acute steroidogenic

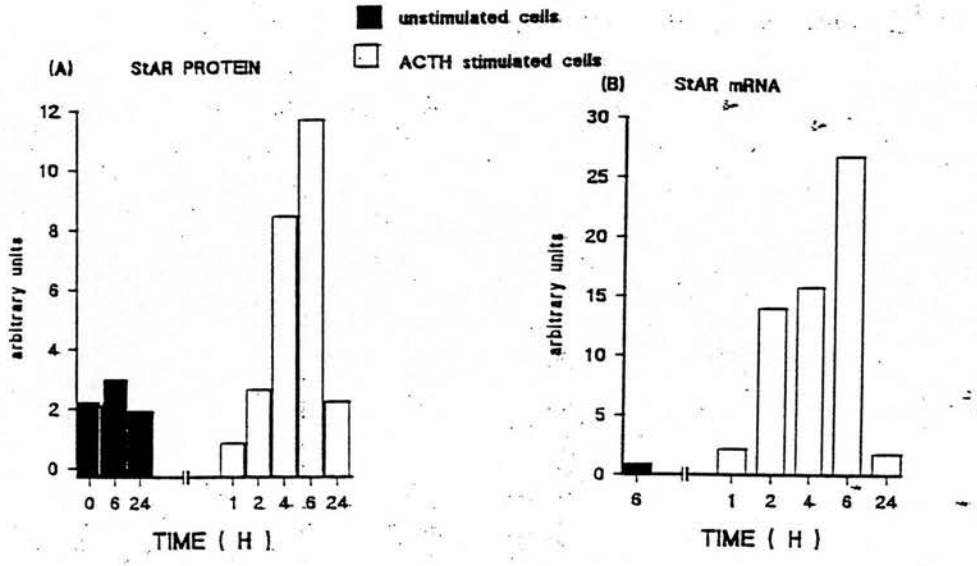

FIGURE 2

BAC cells were cultured for 72 hours after isolation, then incubated with/without ACTH (10^{-8} M) for 1, 2, 4, 6, and 24 hours. (A) Quantification of Western blot analysis (30 kDa band) and (B) Quantification of northern blot analysis (3 kb band) was made using an image analyser.

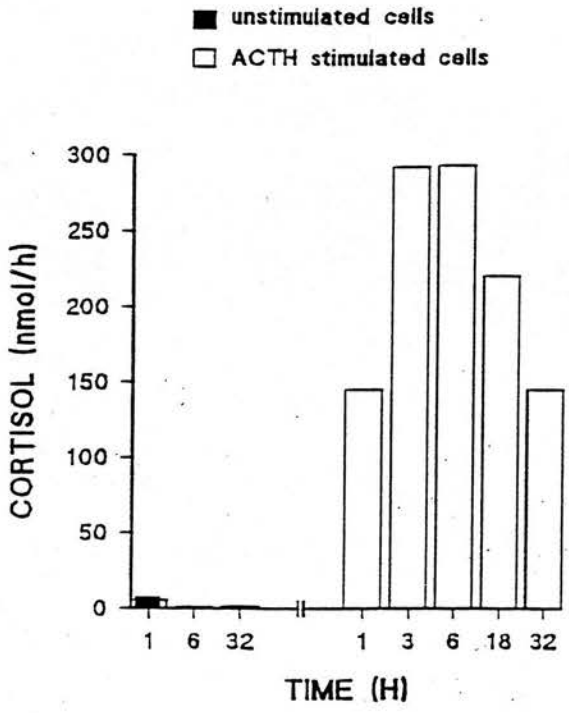

FIGURE 3

Effect of time of ACTH treatment on the rate of cortisol secretion into media overlying BAC cells (nmol/hour)

response, while newly synthesised StAR is needed to maintain steroidogenesis over a period of hours. Consistent with this hypothesis, the decreased rate of cortisol secretion observed after prolonged (24 hour) ACTH treatment is associated with decreased StAR mRNA and protein levels (Figure 3). The discordance between StAR protein and mRNA levels, and the production of cortisol at the early time–points merits further investigation.

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Acute regulation of the bovine gene for the steroidogenic acute regulatory protein in ovarian theca and adrenocortical cells

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ABSTRACT

Upregulation of the steroidogenic acute regulatory protein (StAR) is implicated in the rapid synthesis and secretion of steroidogenic cells to produce steroids in response to stimulation by trophic hormones of the gonadal and stress axes. In the present study, we have assessed the kinetics of both *StAR* gene transcription and protein biosynthesis in primary cell cultures of bovine adrenocortical and ovarian theca cells, under conditions of acute stimulation by corticotrophin (ACTH) and luteinizing hormone (LH), respectively. In both cell systems, detectable upregulation of *StAR* gene transcription occurred within 1–2 h, reaching maxima at 4 h (theca cells) or 6 h (adrenocortical cells). mRNA levels returned rapidly to baseline, by 12 h or 24 h, respectively. Specific StAR protein levels were assessed by western blotting using a novel antibody raised against a bovine StAR peptide, and showed a similar fast upregulation, albeit delayed by 1–2 h compared with the mRNA. The response of the cultured theca cells was more acute than that of the adrenocortical cells, possibly reflecting the propensity of the LH receptor to desensitize rapidly, unlike the ACTH receptor. The primary bovine theca cell cultures were also used for fully homologous transfection studies using various deletion promoter–reporter constructs of the bovine *StAR* gene. Kinetic analysis of the results indicated that the acute transcriptional response resides within the proximal (−315 bp) promoter region, which includes two putative responsive elements for the steroidogenic factor-1. More distal promoter regions may be involved in modulating the specificity of expression by combining enhancer and inhibitory functions.

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INTRODUCTION

In steroidogenic tissues, the rate-limiting step in the acute hormone-dependent upregulation of steroid biosynthesis is considered to be the transport of the cholesterol substrate to the inner mitochondrial membrane, where it is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage complex (reviewed in Thomson 1998). This rate-limiting mitochondrial transport is dependent upon the *de novo* biosynthesis of new protein (reviewed in Stocco & Clark 1996). An important candidate for this role is the recently cloned steroidogenic acute regulatory protein (StAR; Clark *et al.* 1994), which is rapidly and highly upregulated after a steroidogenic stimulus, and becomes associated with mitochondria (Stocco & Clark 1996). Furthermore, the apparently indispensible nature of StAR is revealed by the severe impairment of steroidogenesis after inactivation of the *StAR* gene in mice by homologous recombination (Caron *et al.* 1997*b*), or in the naturally occurring human deficiency disease, congenital lipoid adrenal hyperplasia (Miller 1997).

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The cDNA and gene sequence encoding the StAR protein have been elaborated, initially for the mouse (Clark *et al.* 1994), and subsequently for other species, including the bovine (Hartung *et al.* 1995). Initial experiments investigated the basal levels of StAR mRNA in different tissues *in vivo*, or in tumour cell lines, and the levels attained after long-term stimulation. Although several studies have looked at factors regulating the *StAR* gene promoter (reviewed in Reinhardt *et al.* 1999*b*), most of these used a prolonged (>12 h) stimulation protocol, and so the molecular mechanisms responsible for the acute (<4 h) cAMP-dependent upregulation of *StAR* gene transcription remain unclear. In order to investigate this in a homologous system, we have exploited the ability of bovine ovarian theca cells and bovine adrenocortical cells to produce large amounts of steroids in response to acute stimulation by the natural adenylate cyclase-linked secretagogues, luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), respectively. Using northern and western blotting for StAR mRNA and protein, we first characterized the time-course of the acute specific stimulation of *StAR* gene transcription in these cell types by their natural effectors. Subsequently, we used transfection of previously characterized promoter–deletion–reporter constructs derived from the bovine *StAR* gene (Rust *et al.* 1998) into cultured bovine primary ovarian theca cells to provide a completely homologous system in which to delineate elements of the StAR promoter that are required for the rapid LH-dependent increase in transcription. These results show that the time- and hormone-specificity of *StAR* gene transcription reside within the proximal promoter region, between − 315 and the transcriptional start site.

MATERIALS AND METHODS

Cell culture

Bovine theca interna cells were prepared from large antral follicles (10–25 mm diameter) of ovaries collected from mid- to late-cycle cows at the local abbatoir. Cell preparation and primary culture conditions were exactly as described by Bathgate *et al.* (1999). After Percoll purification, cells were resuspended in 1 : 1 Dulbeccos's minimal essential medium and Ham's F-12 medium, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·1% BSA, 5 μg/ml transferrin, and 5 ng/ml sodium selinite. For mRNA and protein preparation, approximately 10^6 cells were seeded into vitrogen-coated six-well plates and cultured at 37 °C under 95% air–5% CO_2 for 6 days (with medium changed every 2 days), attaining in that time approximately 80% confluence. For measurements of endogenous mRNA and protein production, after the medium change on day 6, cells were supplemented in addition with 100 ng/ml bovine insulin with or without 10 ng/ml bovine luteinizing hormone (bLH; a kind gift of NIADDK and the National Hormone and Pituitary Program, NIH, Bethesda, MD, USA), and culture continued for the times indicated. Primary zona fasciculata/reticularis (ZFR) cells were isolated from sliced bovine adrenocortical tissue as previously described (Williams *et al.* 1989, Nicol *et al.* 1998) and were plated at a density of $1{\cdot}5 \times 10^6$ per well of a six-well plate for both mRNA and protein studies. After plating, cells were maintained for 48 h in Ham's F-10 medium containing 10% v/v controlled processed serum replacement-1 (Sigma, Poole, Dorset, UK), 100 IU penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B at 37 °C under 95% air–5% CO_2, with one medium change after 24 h, and then for a further 16 h in Ham's F-10 medium containing 0·2% BSA and antibiotics as above, before hormone treatment using 10 nM Synacthen (synthetic ACTH(1–24) peptide, Novartis Pharmaceuticals, Camberley, Surrey, UK) in the same medium.

Northern hybridization

For the ovarian theca cells, total RNA was extracted using the peqGOLD RNA-Pure (peqLab, Erlangen, Germany) reagent and pooled from two wells per experiment for each data point. Two micrograms total RNA per slot were subjected to northern hybridization using 1·3% agarose–2·2 M formaldehyde gels in morpholinopropanesulphonic acid running buffer (Sambrook *et al.* 1989). RNA was transferred to a nylon membrane (Nytran; Schleicher & Schüll, Dassell, Germany) by overnight capillary transfer and fixed by u.v. crosslinking. Hybridization with a 750 bp gene-specific probe from the 5′ region of the bovine StAR cDNA was exactly as previously described (Hartung *et al.* 1995). To control for even loading and transfer of the RNA, blots were rehybridized using a radiolabelled probe specific for the bovine S15 ribosomal protein (Bathgate *et al.* 1999). For the ZFR cells, total RNA was isolated using the RNAzol B reagent (Biogenesis, Poole, Dorset, UK), and 20 μg RNA per slot was denatured with glyoxal (Thomas 1983), resolved on a 1·2% agarose gel in 10 mM sodium phosphate pH 7·0 buffer, capillary-transferred to positively charged nylon membranes (Stratagene) and fixed by u.v. crosslinking. Hybridization for StAR mRNA was as above, but using a somewhat

shorter 5′ cDNA probe (accession number S79908, nucleotides 210–608). Even loading and transfer were confirmed by rehybridization using a β-actin probe. All northern and western (see below) blotting experiments were repeated at least twice for fully independent batches of cells, and gave fully reproducible results.

Antibody production and western blotting

Total cellular protein (ZFR cells 25 μg, theca cells 15 μg per sample), was prepared by homogenization in PBS containing 0·1% SDS and 1% sodium deoxycholate, resolved on a 12% acrylamide gel, and electroblotted onto Immobilon-P membrane (Sigma). Western analysis was carried out using, as primary antibody, a polyclonal sheep antiserum raised against a peptide fragment (AMQRALGIL KDQEGWKKESRQANGDE; amino acids 82–107; Hartung *et al.* 1995) from the predicted bovine StAR protein sequence attached to a lysine-web-based eight-branched antigen scaffold (The Binding Site, Birmingham, UK), and a donkey anti-sheep horseradish peroxidase-conjugated second antibody. After preliminary optimization (data not shown), primary and secondary antibodies were used respectively at 1 : 10 000 and 1 : 25 000 dilutions in PBS containing 10% blocking buffer (Pierce, Rockford, IL, Staffs, USA) and 2% w/v non-fat milk (Marvel Original; Premier Beverages, Stafford, UK) for immunodetection of adrenocortical proteins, and at 1 : 7500 and 1 : 10 000 dilutions, respectively, in PBS containing 10% blocking buffer (Pierce) and 1% non-fat milk for theca cell proteins. The final signal was visualized by chemiluminescence (SuperSignal ULTRA kit; Pierce). The specificity of the antibody was demonstrated first by its ability to detect a protein in adrenocortical protein extracts at the anticipated size of approximately 30 kDa, the pattern of expression of which followed closely behind that of the StAR mRNA (see later), and, secondly, by the complete elimination of the specific 30 kDa band as a result of the addition of an excess (>3 ng/ml) of the peptide used to generate the antibody (not shown).

Cell transfection and analysis

All bovine StAR promoter–reporter DNA constructs, in addition to control vectors, were exactly as previously described (Rust *et al.* 1998). DNA was purified using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) as described by the manufacturer. For transfection experiments, 10^6 bovine theca cells per well, prepared as above, were seeded into 12-well plates, and cultured as above in the presence of 100 ng/ml insulin for 2 days. Cells were then washed briefly in PBS before the addition of OPTI-MEM (Gibco-BRL, Deisenhofen, Germany) transfection medium. Transfection was carried out by adding 5 μg per well LipofectAMINE (Gibco-BRL) at a ratio of 2 : 1 with the DNA to be transfected, and incubating for 5 h. Medium was then replaced by the standard culture medium and incubation continued for a further 3 days, before treatment or not with 10 ng/ml bLH, for the times as indicated. Cells were then washed rapidly in PBS and immediately extracted into 40 μl reporter lysis buffer (Luciferase Assay System; Promega, Madison, WI, USA) before luciferase activity was measured using the same kit. As cotransfected control, a β-galactosidase reporter gene driven from the cytomegalovirus (CMV) promoter was used, measuring the resulting activity using the Galacto-Light kit (Tropix, Bedford, MA, USA). All transfections were performed in triplicate for any batch of primary cells. All experiments were repeated at least twice using independent batches of cells, with fully reproducible results.

RESULTS

Expression of endogenous StAR mRNA and protein in primary cultures of bovine ovarian theca and adrenocortical (ZFR) cells

The endogenous StAR mRNA in bovine ovarian theca and adrenocortical (ZFR) cells was expressed as transcripts of three different sizes (Figs **1**, 2), as shown previously also for bovine corpus luteum (Hartung *et al.* 1995, Pescador *et al.* 1996) and predicted from the positions of alternative polyadenylation sites within the 3′ UTR of the cloned bovine cDNA (Hartung *et al.* 1995). These migrated as two major bands at approximately 3·0 kb and 1·8 kb, and a minor band at 1·6 kb, evident only in the theca cells. Levels in untreated cells were very low for both cell types (Figs **1**B, 2B). After treatment of theca cells with bLH or adrenocortical cells with ACTH, there was a similar rapid increase in the relative amounts of the two major StAR transcripts in both cell systems. This increase was already detectable after 1 h of stimulation, by comparison with the untreated controls, and reached a maximum at about 4 h (theca cells, Fig. 1A) and 6 h (adrenocortical cells, Fig. 2A) of treatment. At the time of maximal induction of StAR mRNA, the shorter 1·6 kb transcript became readily detectable in the theca cell cultures (Fig. 1A). Also of significance was the rapid decline in transcript levels at subsequent

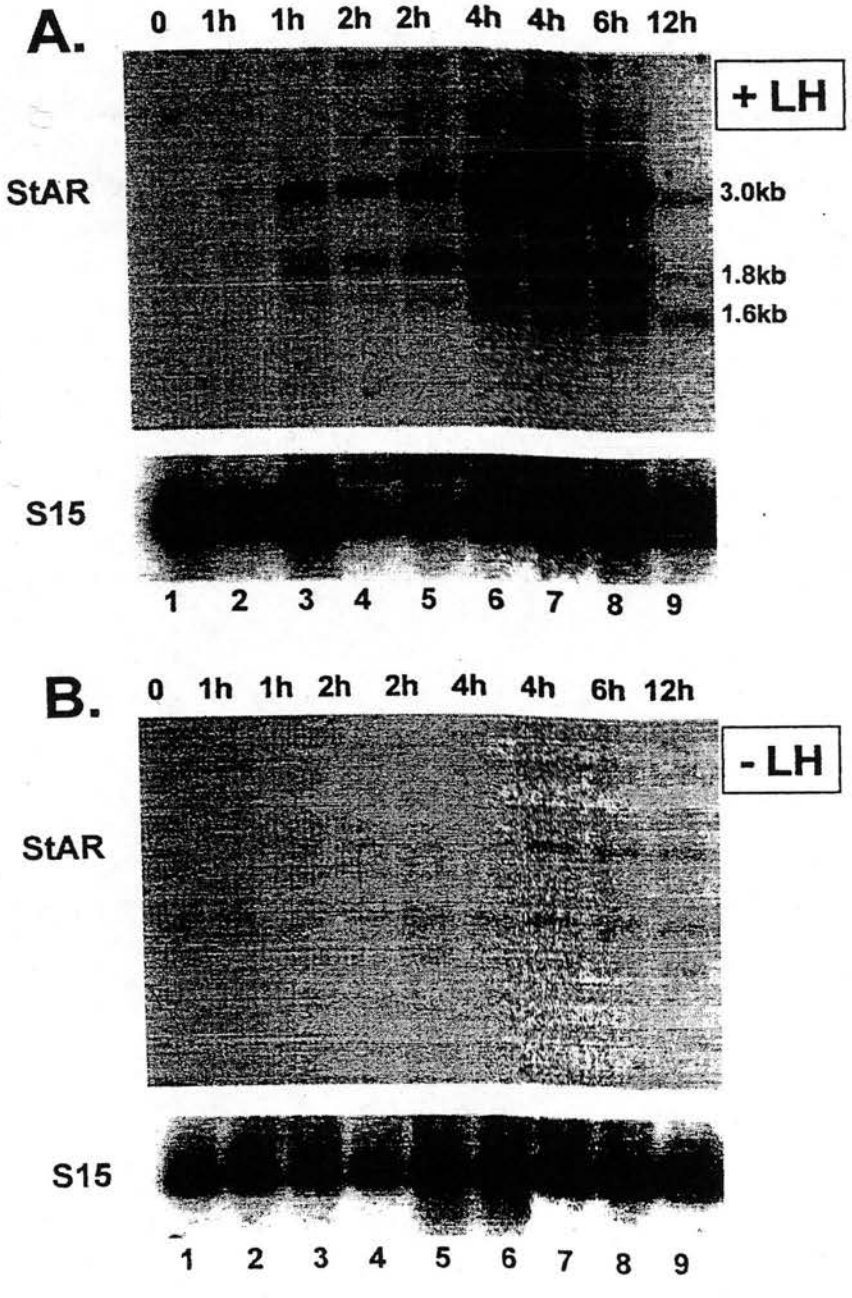

FIGURE 1. Northern hybridization of StAR mRNA in extracts of bovine theca cells cultured for the time indicated in the presence (A) or absence (B) of 10 ng/ml LH. As control for even loading and transfer, blots were rehybridized against a probe for the bovine ribosomal protein, S15. The blots in (A) and (B) are from parallel experiments using the same batch of cells. Duplicate lanes represent RNA from independent parallel cell cultures from within the same experiment, to indicate the extent of within-batch variation.

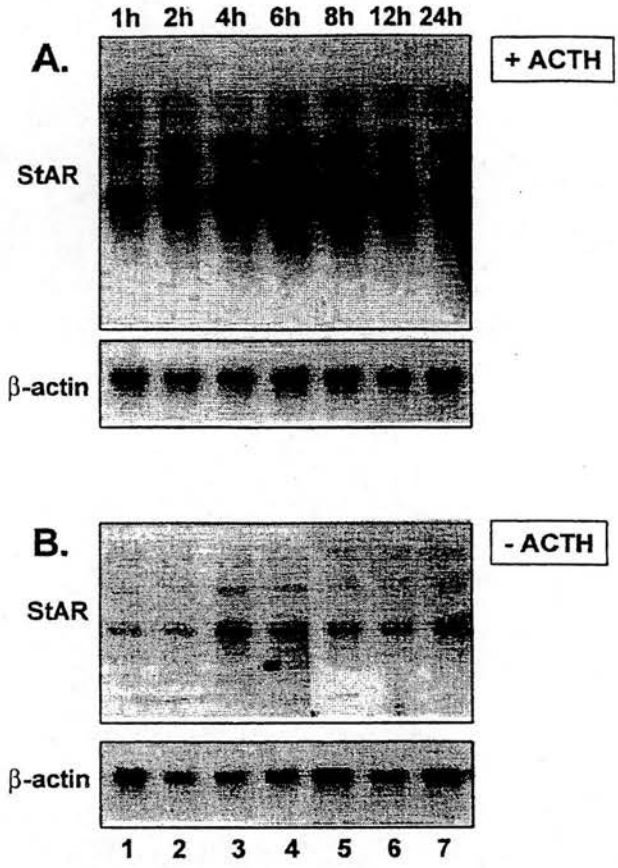

FIGURE 2. Northern hybridization of StAR mRNA in extracts of bovine adrenocortical (zonae fasciculatis and reticularis) cells cultured for the times indicated in the presence (A) or absence (B) of 10 nM ACTH. As control for even loading and transfer, blots were rehybridized against a probe for β-actin. The blots in (A) and (B) are from parallel experiments using the same batch of cells, with all operations being performed in parallel.

time-points, such that, at 12 h (theca cells) or 24 h (adrenocortical cells), StAR mRNA had returned to basal (unstimulated) levels. The apparent and slight long-term increase in StAR mRNA in the absence of bLH (Fig. 1B) probably reflected the differentiation of these cells in culture under the influence of insulin only (Bathgate *et al.* 1999).

Total cellular protein was also prepared from similar cultures of stimulated and unstimulated cells and subjected to western blotting using a newly developed anti-StAR polyclonal antibody. This antibody was raised against a 26-mer sequence from the predicted external surface of the bovine StAR protein (Hartung *et al.* 1995) and specifically recognized a 30 kDa protein (Fig. 3). Protein bands of similar size were detected in adrenocortical and luteal protein extracts using another polyclonal antibody raised against mouse recombinant StAR protein (Pescador *et al.* 1996, Ronen-Fuhrmann *et al.* 1998, and data not shown). The time-course of changes in StAR protein levels in the two cell types was similar (Figs 3, 4), with a first detectable increase at 2 h and maximum levels being maintained through to 6 h (theca cells) or somewhat longer (adrenocortical cells), but with a return to near basal values by 12–24 h. Thus StAR protein production was marginally delayed, both in appearance and disappearance, with respect to the changes in levels of the specific mRNA. Absolute levels of StAR protein, both basal and stimulated, appear to be much greater in the adrenocortical cells than in the bovine theca cells, as indicated by the control lanes (1 and 2) in Fig. 3, in which the same amount of total protein as in the other lanes had been loaded.

Acute regulation of the bovine StAR gene promoter in homologous cell culture

In a previous study (Rust *et al.* 1998), we have been able to characterize the role of certain *cis* elements within the upstream promoter region of the bovine *StAR* gene responsible for expression in a heterologous cell system cotransfected or not with the transcription factor, steroidogenic factor-1 (SF-1; Ad4 BP; Fig. 5). In this system, deletion promoter–reporter constructs had been transfected into non-steroidogenic cells together with appropriate expression vectors. In order to determine whether the SF-1-responsive elements are also involved in endogenous *StAR* gene expression in primary cultures of bovine steroidogenic cells (i.e. in a homologous system as close to the *in vivo* situation as possible), and are sufficient to account for the acute up- and downregulation of the *StAR* gene, a time-course for activation of defined promoter–reporter constructs was determined under acute bLH stimulation after transfection into primary ovarian theca cells (Fig. 6). The results show that there was consistently detectable upregulation in response to bLH by 3 h of stimulation. The maximum level of luciferase activity accumulated in the cells was reached by 4–6 h, and was not further increased at later times. A comparable time-course was obtained irrespective of the use of a long promoter fragment (−1245 bp; Fig. 6A), or a shorter fragment (−315 bp; Fig. 6B) attached to the luciferase reporter gene. However, it should be noted that, in repeated experiments, the response to LH appeared generally to be more robust, consistent, and somewhat earlier in these primary cell

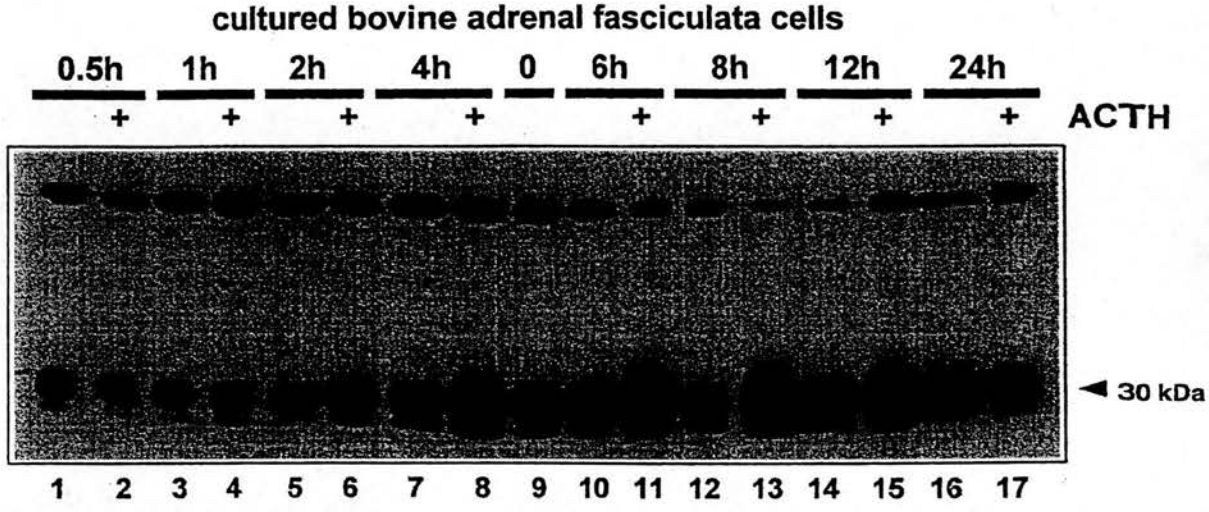

FIGURE 3. Western blot of immunoreactive StAR protein in extracts of bovine adrenocortical cells cultured as indicated for increasing times in the absence or presence (+) of 10 nM ACTH. The upper, more slowly migrating band appears to be non-specific and its intensity does not alter with time or incubation conditions. The specific, approximately 30 kDa StAR protein appears as a doublet with a more rapidly moving isoform increasing in intensity, in parallel with the principal StAR protein band. In all lanes, 25 μg protein were loaded.

cultures, for the shorter −315 bp construct (cf. Fig. 7).

Experiments using different promoter–deletion constructs transfected into theca cells optimally stimulated by bLH for 4 h indicated that the acute stimulation of the bovine *StAR* gene requires only the minimal promoter up to −315 bp (Fig. 7), which includes the first two proximal SF-1 binding motifs (Rust *et al.* 1998). Indeed, even shorter constructs comprising only one (−203) or none (−101) of the proximal SF-1 sites, also appear to be responsive to LH. Longer promoter constructs, however, do not increase the level of luciferase activity obtained. As already indicated in Fig. 7, these appear to have reduced activity by comparison with the shorter constructs. This would suggest that the region upstream of the −315 construct may include a negative factor that could modulate the activity of the proximal promoter.

DISCUSSION

The acute response of steroidogenic cells to the anterior pituitary hormones, LH or ACTH, is an essential part of the regulatory mechanisms promoting steroid biosynthesis. Both peptide hormones are released in short pulses or trains of pulses, and elicit both immediate and sustained effects. Amongst the immediate effects are those influencing steroid secretion (reviewed in Thomson 1998). In addition to such very acute events, there is also an effect a the transcriptional level. This effect was quite rapid newly transcribed StAR mRNA being detectable in both adrenocortical and ovarian thecal cells after about 1 h of stimulation. Equally important however, is the observation, also for both cell types that levels of mRNA peaked at about 4–6 h and then declined to basal values by, maximally, 12–24 h. This shows that the stimulatory effect upon transcription is short-lived, and that mechanisms are present in these steroidogenic cells for the rapid degradation of the newly synthesized StAR mRNA. It is important to note that the primary cell cultures were exposed continuously, through the treatment period, to the effectors LH or ACTH. It is known that the LH receptor is rapidly downregulated (desensitized, internalized) within the first few minutes of hormone exposure (e.g. for bovine luteal cells; Budnik & Mukhopadhyay 1987), thus all subsequent LH-dependent events will be receptor-independent consequences of this initial stimulation. For the effect of ACTH on adrenocortical cells, the situation appears to be different. Instead of being desensitized, the cognate receptor appears rather to be upregulated (Penhoat *et al.* 1989). This could explain the more sustained transcriptional response of the *StAR* gene to ACTH treatment evident in the adrenocortical cell cultures.

By transfecting different promoter–reporter constructs into ovarian theca cells, under a treatment paradigm similar to that above in which we have

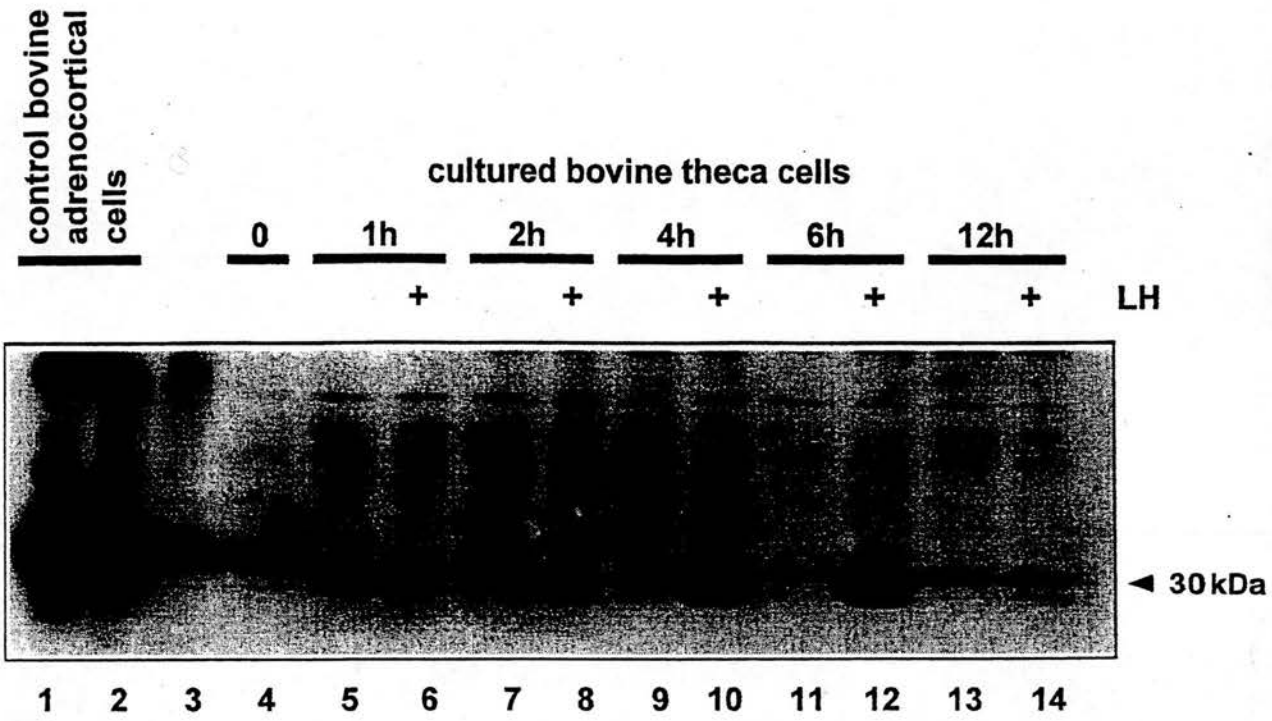

FIGURE 4. Western blot of immunoreactive StAR protein in extracts of bovine theca cells cultured as indicated for increasing times in the absence or presence (+) of 10 ng/ml bovine LH. It should be noted that the absolute levels of the StAR protein are considerably less than in the adrenocortical cells (lanes 1 and 2), and there is thus an increased non-specific background. As for the adrenocortical cells, the StAR protein appears to be represented as a doublet. In all lanes, 15 μg protein were loaded.

shown acute upregulation of the endogenous *StAR* gene, we were also able to show that luciferase activity (i.e. expression of active protein) follows the same time-course of induction as the native StAR protein. Increased luciferase activity was evident first at 2–3 h, just like the endogenous StAR protein seen in the western blots, suggesting that both StAR and luciferase mRNAs are translated in a comparable manner. The induction of reporter activity was, as expected, slightly delayed by comparison with the induction of transcription. The disappearance of the luciferase activity will probably have a

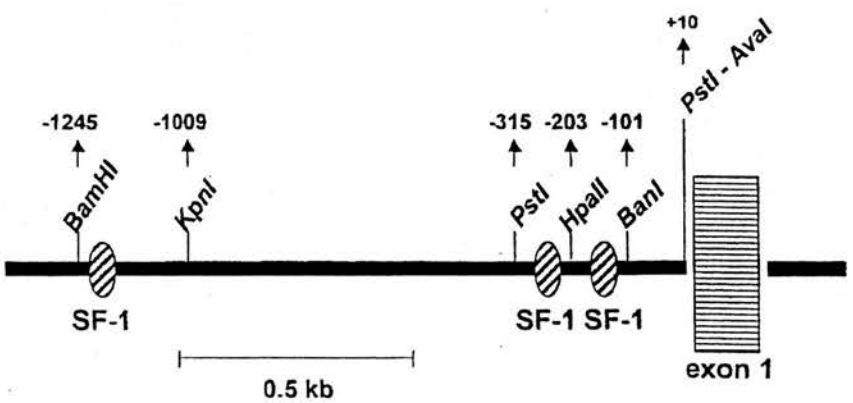

FIGURE 5. Schematic representation of the bovine *StAR* gene and its promoter, indicating putative transcription factor binding elements.

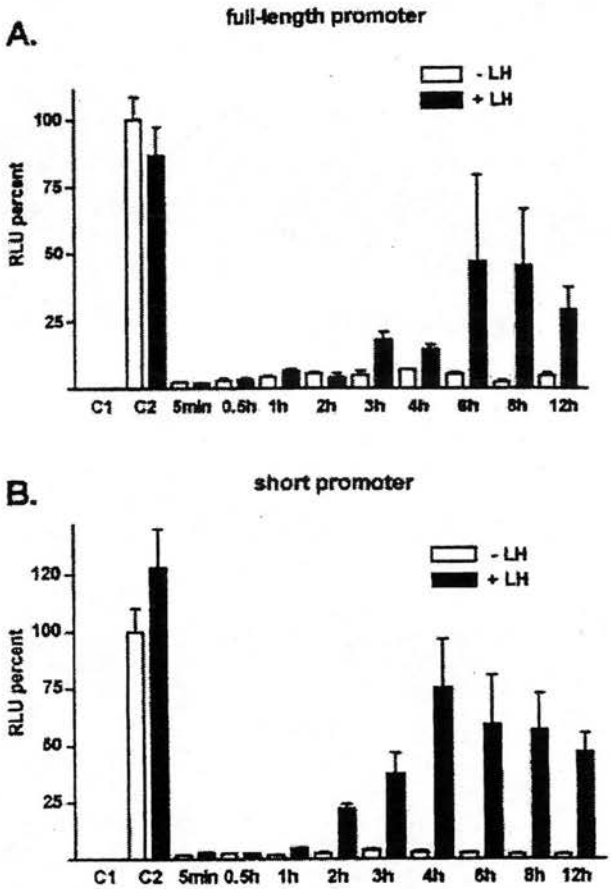

FIGURE 6. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with either the −1245 (full-length, A) or the −315 (short, B) bovine *StAR* gene promoters. Three days after the transient transfection, cells were stimulated or not as indicated by addition of 10 ng/ml bovine LH. The amount of luciferase protein synthesized is estimated from its enzymatic activity expressed in relative light units (RLU; relative to the cotransfected CMV-β-galactosidase constitutive construct) as a percentage of the level attained with the strong constitutive promoter of the pGL3-C construct (C2) in the absence of LH. C1, luciferase activity of the empty reporter vector, pGL3-B. Data are expressed as means ± S.E.M from parallel triplicate experiments using the same batch of primary cells.

time-course different from that for the endogenous protein, as this will depend on the function of substrate-specific proteases. Nevertheless, it is evident that there is a plateau in luciferase expression at 4–6 h, with no further increase after this, reflecting precisely the short phase of transcriptional induction. This experiment showed that the promoter information required for this acute

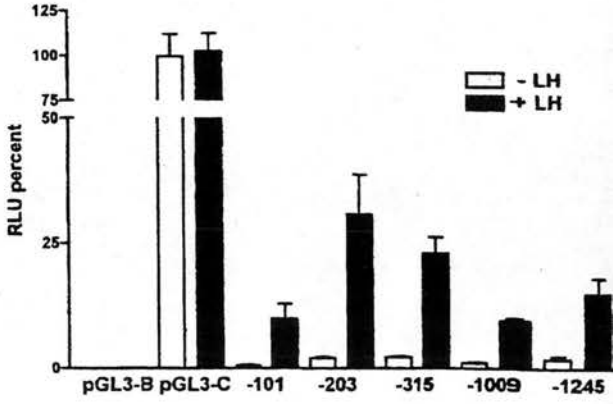

FIGURE 7. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with different deletion fragments of the bovine *StAR* gene promoters. The promoter fragments correspond to DNA sequences extending upstream from the transcription start site to the restriction sites for BanI (−101), HpaII (−203), PstI (−315), KpnI (−1009) and BamHI (−1245), respectively. Results are expressed in relative light units (RLU) as a percentage of the luciferase activity obtained using the strong constitutive promoter construct, pGL3-C, in the absence of LH.

upregulation of transcription resides within the firs 1200 bp upstream of the transcription start site, an probably within the first 315 bp of this. Use promoter–deletion constructs confirmed what w have previously shown for this gene using heterologous transfection system (Rust *et al.* 1998 namely that LH-induced, cAMP-dependent gen activation is maximal where the first two SF-1 responsive elements, which reside within th −315 bp immediate upstream region of the pro moter, are present. It should be noted, though, tha a marked LH-dependent stimulation was als evident for the very short −101 construct (Fig. 7 which does not include an SF-1 binding elemen suggesting that SF-1 may not be absolutel required to mediate the stimulatory effect of LH Other genes have also been described in the same c related cell types, for example, $P450_{SCC}$ in lutes cells (Liu & Simpson 1997) or oxytocin in granulos cells (Wehrenberg *et al.* 1994), which also depen upon SF-1 interaction, and respond to LH stimulation. These responses, however, have a quit different time kinetic and cell specificity. Thu other factors must act in conjunction with SF-1 t encode the cellular and temporal specificity ob served for the *StAR* gene. One candidate in th case of the mouse *StAR* gene is the transcriptio factor, CCAATT/enhanced binding protein (C/EBPβ) (Reinhardt *et al.* 1999*a*, Silverma

et al. 1999). Support for this view is provided by a comparison of the present transfection results, using cells endogenously expressing the *StAR* gene, with the heterologous system used previously. In the previous study, the transcriptional activity induced by SF-1 attained maximally only 2% of the level reached by the same constitutively active control plasmid (pGL3-C) as that used here; this compares with greater than 50% in the present homologous system. Similarly, a constitutively active protein kinase A subunit was able to increase the SF-1-dependent transcriptional activation by only about 50% in the previous study whereas, in the homologous system, LH induced up to a 10-fold increase in reporter gene activity. The molecular mechanisms by which SF-1 could be involved in cAMP-dependent signal transduction are not yet understood. In a recent study, a potential serine acceptor site for protein kinase A (PKA) phosphorylation, and the C-terminal activation domain were both implicated in the transcriptional upregulation of the high-density lipoprotein receptor gene in rat luteal cells via SF-1 (Lopez *et al.* 1999). Also, possible mitogen-activated protein kinase phosphorylation of SF-1 has been suggested in the context of cofactor recruitment (Hammer *et al.* 1999).

The requirement for other factors to act with SF-1 in mediating cAMP-regulated signal transduction would also offer an explanation for the anomalous findings regarding specific nuclear protein-binding to the bovine StAR promoter (Rust *et al.* 1998). Of the three putative SF-1 binding motifs evident in the bovine StAR promoter (Fig. 5), only the distal element bound purified SF-1 with high affinity. The second proximal element bound SF-1 only weakly, and the most proximal element failed to show any binding activity (Rust *et al.* 1998). Yet it is the proximal promoter containing the first SF-1 motifs which, in this and other studies, appeared to mediate SF-1-dependent activation of the *StAR* gene by adenylate cyclase in (for example) humans (Sugawara *et al.* 1996), mouse (Caron *et al.* 1997*a*), rat (Sandhoff *et al.* 1998) and pig (LaVoie *et al.* 1999). Findings of a recent study using the mouse *StAR* gene promoter even suggested that an interaction of SF-1 in the proximal promoter may not be essential, and that adenylate cyclase-induced activation can be mediated by a combination of the transcription factors C/EBPβ and GATA-4 only (Silverman *et al.* 1999). This opinion is supported by findings of the present study, in which the −101 construct of the bovine *StAR* gene was able to transduce LH-stimulation to the reporter gene. This fragment does include an inverse GATA motif at position −65 (Rust *et al.* 1998).

Other regions of the promoter may be involved in the definition of expression specificity. There is evidence, from the transfection studies, for a mildly inhibitory element in the region upstream of nucleotide −315. There is also, in this region, marked nuclear protein binding unrelated to SF-1 (Rust *et al.* 1998). Furthermore, although not apparently contributing to gene activation, a very good binding site for SF-1 exists in the upstream region of the promoter at −1100 (Rust *et al.* 1998). Concerning the rapid switching off of the *StAR* gene, this might involve the activation of the negative transcription factor, DAX-1, which has been shown to interact with the mouse *StAR* gene promoter, possibly interfering directly with SF-1 binding in the proximal promoter region (Zazopoulos *et al.* 1997, Reinhardt *et al.* 1999*b*, Sandhoff & McLean 1999).

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